For submission to Psychoneuroendocrinology

Stress-induced behavioral and metabolic adaptations lead to an obesity-prone phenotype in individuals with elevated cortisol responses

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Abbreviated title: Metabolic and behavioural responses to stress

Keywords: Hypothalamo-Pituitary-Adrenal Axis, Lipopolysaccharide, Thermogenesis, Energy balance

The underlying cause of predisposition to obesity is complex but one marker is cortisol responsiveness. Selection of sheep for high (HR) or low (LR) cortisol responses to adrenocorticotropin shows that HR are more likely to become obese. Increased propensity to obesity is associated with reduced skeletal muscle thermogenesis. We sought to determine whether metabolic or behavioural responses to stress also contribute to altered propensity to obesity in LR and HR. Animals (n=5-10/ group) were exposed to 3 stressors and we measured food intake and thermogenesis. Thermogenesis was recorded with dataloggers implanted into muscle. Stressors were hypoglycaemia (0.125units/kg insulin, i.v.), a barking dog and immune challenge (200ng/kg Lipopolysaccharide–LPS, i.v.). LR animals showed a greater catabolic state in response to both immune and psychosocial stressors. LPS reduced ($P<0.01$) food intake in both groups but LR showed a greater ($p<0.05$) reduction in food intake and a more substantial ($p<0.05$) rise in muscle temperature. Introduction of the barking dog reduced ($P<0.05$) food intake in LR only. These metabolic differences coincided with differences in cortisol responsiveness, where HR animals had increased ($P<0.05$) cortisol in response to both immune and psychosocial stressors. In addition, we assessed behaviour in the following paradigms: 1. Isolation in the open field test, 2. Response to a human intruder and 3. Food competition. LR had greater ($p<0.05$) activity, reduced fearfulness and displayed a proactive coping style of behaviour. Thus we demonstrate that high cortisol responsiveness identifies animals with stress-induced metabolic and behavioral traits that may contribute to susceptibility to obesity.

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Introduction

There is a strong nexus between the hypothalamo-pituitary-adrenal (HPA) axis and energy homeostasis. A number of studies in rodents and humans have demonstrated that stress can either increase or reduce food intake (Epel, Lapidus et al. 2001, Pecoraro, Reyes et al. 2004, Adam and Epel 2007, Jahng, Kim et al. 2008, Tomiyama, Dallman et al. 2011). Hallmark studies in rats by Dallman and colleagues demonstrate that stress increases the preference for palatable foods that are high in fat and sugar (Pecoraro, Reyes et al. 2004, Dallman, Pecoraro et al. 2005, Dallman 2010, Tomiyama, Dallman et al. 2011). This led to the 'comfort' food hypothesis, stating that increased consumption of foods high in fat and sugar leads to feedback on the HPA axis to dampen the stress-induced elevations in circulating corticosteroid levels (la Fleur, Houshyar et al. 2005).

In addition to the availability of palatable foods, effects of stress on feeding are influenced by the type of stressor as well as the severity and duration of the stressor (Valles, Marti et al. 2000, Solomon, Foster et al. 2007, Calvez, Fromentin et al. 2011). On the other hand, a number of studies have demonstrated individual variation in cortisol responsiveness in response to the same stress can impact on the metabolic sequelae. Thus in any given population, high or low cortisol responder individuals can be identified (Epel, Lapidus et al. 2001, Touma, Bunck et al. 2008, Tomiyama, Dallman et al. 2011, Lee 2013). Cortisol responsiveness is also a strong determinant of the feeding response to stress, whereby subjects characterized as high cortisol responders (HR) tend to eat more in response to stress than do low cortisol responders (LR) (Epel, Lapidus et al. 2001, Adam and Epel 2007, Tomiyama, Dallman et al. 2011). Importantly, our work in sheep (Lee, Clarke et al. 2014) demonstrates that high cortisol responsiveness confers a predisposition to obesity. This increased propensity to weight gain was primarily associated with a reduction in thermogenesis in skeletal muscle, without an attendant change in food intake.

In addition to the change in thermogenesis, we propose that altered propensity to obesity in LR and HR may also be associated with ancillary factors such as altered metabolic responses to stress and/or innate differences in temperament and

behavior. In humans, obesity leads to differences in temperament, which manifest as low inhibitory control (Anzman and Birch 2009), impulsiveness (Sullivan, Cloninger et al. 2007) and calmness (Wells, Stanley et al. 1997). Indeed, in sheep, adipose tissue thermogenesis is lower in animals selected for a calm temperament compared to those selected for a nervous phenotype (Henry, Blache et al. 2010). It has also been hypothesized that innate differences in coping strategy (e.g. reactive compared to proactive) may impact on energy expenditure and metabolism (Garland, Schutz et al. 2011). Accordingly, the current study sought to examine the link between behavioral and metabolic responses to stress and the altered propensity to obesity in animals characterized as LR and HR. We demonstrate that LR animals show greater negative energy balance in response to both immune and psychosocial stressors. Furthermore, LR animals showed increased activity, fearlessness and a proactive coping strategy under several behavioral paradigms. This suggests, that the increased propensity to obesity in HR is underpinned by an innate reduction in energy expenditure as well as distinct behavioral and metabolic coping strategies in response to stress.

Materials and Methods

Selection of Low and High Cortisol Responding Sheep

This work was performed in corriedale ewes and was approved by the Monash Animal Research Platform animal ethics committee. Prior to the selection of LR and HR and prior to each experiment, reproductive status was standardized; the estrous cycles of the ewes were synchronized by an i.m. injection of 125µg Cloprostenol and testing was carried 7 days later during the luteal phase of the cycle. Animals (n=100) were challenged with an i.v. injection of synthetic ACTH (Synacthen, Novartis, North Ryde, NSW, Australia) to select high (HR) and low (LR) cortisol responders as described previously (Lee, Clarke et al. 2014). In brief, outbred ewes received an indwelling jugular venous cannula, which were kept patent with heparinized saline (100KIU/ 1L). Cannulae were used for sampling and for injection of Synacthen (0.2µg/kg body weight). Samples were collected at -60, -30, 0, 30, 60 and 90 min relative to injection and centrifuged immediately at 3000rpm at 4°C. Plasma was

harvested and stored at -20°C until assayed for cortisol (see below) and data were analyzed by calculating the area under the curve (AUC). After the initial challenge, 20% at each extreme (high and low) were selected and the synacthen test was repeated to ensure veracity of groups. After the second challenge 10% of animals were selected as either a LR or HR (n=10/ group).

Plasma cortisol was measured by radioimmunoassay as described previously (Bocking, McMillen et al. 1986). For the selection of LR and HR cortisol responsiveness was determined by calculating the AUC. Across all of the cortisol. The sensitivity of the assay was 0.3 ng/ml. The intra-assay coefficient of variation was 4.2% and the inter-assay coefficient of variation was 3.2% at 12 ng/ml and 4.5% at 80 ng/ml.

Experiment 1: Episodic profiling of cortisol secretion in HR and LR

To measure basal cortisol secretion blood samples were collected at 10min intervals for 6 hours (0900-1500h). Samples were used to measure plasma levels of cortisol as outlined above. To characterize the secretory profile we performed deconvolution analysis and measured the number of pulses, mode (time taken from the onset of a pulse to reach the peak: min), basal secretion (ng/ml), pulsatile secretion (ng/ml), total secretion (ng/ml), pulse mass (ng/ml) and approximate entropy (ApEn) (Veldhuis, Iranmanesh et al. 1989, Veldhuis, Johnson et al. 1990). In addition, the AUC was assessed across the first hour of sampling (0900-1000h).

Experiment 2: Effect of stress on food intake, thermogenesis and plasma cortisol levels in LR and HR

Part A: Insulin-induced hypoglycaemia

Two weeks prior to experimentation dataloggers (SubCue, Calgary, Canada) were implanted into the skeletal muscle (vastus lateralis) of the hindlimb as previously described (Henry, Dunshea et al. 2008, Henry, Andrews et al. 2011, Clarke, Lee et al. 2012), to monitor tissue temperature. Dataloggers were set to record temperature at 15min intervals. In addition to characterizing thermogenesis, food intake was

measured across the experimental period. Baseline food intake was established across the week preceding the onset of experiments. Animals were fed 2kg of lucerne chaff at 0900h and refusals were weighed to determine daily intake.

Hypoglycaemia was induced by an iv bolus injection of insulin (Actrapid: Novo Nordisk Pharmceuticals, Baulkham Hills, NSW). Blood samples (7ml) were collected between 0900-1500h and insulin (0.125U/kg body weight) was injected at 1200h. Sampling then continued for 3h. This dose was established in earlier work (Knott, Cummins et al. 2010). Cortisol concentrations were measured in all plasma samples. Selected samples (-30, -20, -10, 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180 min relative to insulin injection) were used to measure plasma glucose concentrations, using a YSI2300 STAT glucose/lactate analyzer (Yellow Springs Instrument Co., USA). The measurable range for blood glucose was 0–30mmol/l.

Part B: Immune Challenge

This experiment was carried out following the protocol outlined for experiment 2 except that the animals were treated with LPS (200ng/ kg body weight). This dose was based on earlier studies (Briard, Dadoun et al. 2000, Elsasser, Kahl et al. 2004). Food intake, muscle thermogenesis and plasma cortisol concentrations were measured. In addition, hourly blood samples were used to measure plasma levels of tumour necrosis factor α (TNFα), interleukin-4, -6, -10 and -12, using ovine-specific enzyme-linked immunosorbent assays (ELISA) as described previously (Hope, Kwong et al. 2002, Kwong, Hope et al. 2002, Rahman, Snibson et al. 2004, Hope, Kwong et al. 2005, Abeynaike, Meeusen et al. 2010).

Part C: Psychosocial stress

To induce psychosocial stress, animals were exposed to a barking dog (12:00h) for 5 min. The dog was guided to move from pen to pen whilst continuously barking, however, direct physical contact was prevented (Pierce, Hemsworth et al. 2008). Food intake, muscle thermogenesis and plasma cortisol concentrations were measured as outlined in Experiment 2. Additional blood samples were collected at -10, 0, 10, 30, 50 and 70 min into tubes containing an inhibitor mix (30mg/mL

reduced glutathione and 95mg/mL EGTA) for subsequent measurement of catecholamines. Plasma levels of adrenaline, noradrenaline and dihydroxyphenylglycol (the deaminated metabolite of adrenaline and noradrenaline) were measured by high performance liquid chromatography and coulometric detection as previously described (Lambert and Jonsdottir 1998, Tilbrook, Rivalland et al. 2008).

Part D: Statistical Analyzes

The AUC of cortisol response as well as deconvolution data were analyzed using Student's unpaired t-test. Changes in glucose, cortisol (Experiments 2-4), cytokine, catecholamine levels and temperature were analyzed by repeated measures ANOVA and *post-hoc* comparisons were made using the Bonferroni test. Data are presented as means±SEM and $P<0.05$ was considered significant.

Experiment 3: Behavioral phenotype of LR and HR

All behavioral tests were carried out in the non-breeding season to prevent confounding effects of changes in ovarian steroids. In each experiment, except for the food competition assay, animals were fed *ad lib* prior to the onset of study. Behavior was recorded by video camera and was subsequently analyzed in a blind fashion.

Part A: Open field test

This was carried out to characterize behavioral responses to isolation. The test was conducted in an area of 5 x 3m area divided into 6 equal-sized regions. The enclosure was surrounded by a 1.5m enclosed fence. Single animals were exposed to the area for 5 min during which time behavior was recorded. Recordings were subsequently used to measure the following indices:

Locomotor activity:- the number of times the animal crossed from one of the 6 regions to another.

Vocalisation:- number of bleats

Tunneling:- number of attempts to bury their head under the arena entry/exit gate or fence.

Scratching:- number of times the animals investigated the entry/exit gate by scratching or knocking.

The summation of these active behaviors (omitting locomotor activity) provided the total activity score. All data was analyzed using the non-parametric test, Mann-Whitney U test.

Part B: Arena Test

The arena test was used to characterize fearfulness in response to the presence of a human. The animals entered an arena (5 x 3 m) that was enclosed by 3 walls. At the open end of the arena 2 flock mates were located in visible and auditory contact with the test animal. The human stood between the test subject and it's conspecifics. The arena was equally divided into 3 areas, with zone 1 being the closest to the human observer and zone 3 being the furthest. Behavior of the test animal was recorded for 5 min. We quantified the amount of time each animal spent in zone 1, 2 or 3 as well as the time spent attempting to tunnel under the gate to actively reach its flock mates. Spending time in Zone 1 facing the human intruder was deemed to be less fearful than standing further away in Zone 2 or Zone 3. All data were analyzed using a Mann-Whitney U test.

Part C: Food competition assay

The food competition test was used to examine the competitiveness of an animal in getting to a food source. Prior to the food competition test all animals were fasted overnight. We used the test to mark the aspect of a proactive coping style which is to demonstrate initiative, as oppose to reactive coping style which tends to be more passive. The test was carried out by placing either a HR or LR ewe in a pen next to a randomly selected control animal. At t = 0, the pen doors open and both sheep were allowed to move down a corridor measuring 13m in length. A trough of food (chaff and lupin grain) was placed at the end of the corridor, which was 0.8m wide. Animals were habituated to this environment for 2 days prior testing, to ensure that they were aware of the food source location. The latency of time it took for each test animal to reach the food and begin eating was measured and analyzed relative to the latency for the control. Results were analyzed using an unpaired Student's t-test.

Results

Selection of High and Low Cortisol Responding Sheep

Cortisol responses to ACTH were greater ($P<0.01$) in HR than in LR as assessed by AUC (Figure 1a). Of 100 outbred animals, 10% were selected as HR and LR (n=10/group).

Experiment 1: Episodic profiling of cortisol secretion in LR and HR.

At the time of experimentation baseline food intake was similar in LR and HR (LR: 1.4± 0.2 kg/day versus HR: 1.6±0.2 kg/ day). Deconvolution analysis of the cortisol data showed that the number of pulses, basal secretion, pulsatile secretion, total secretion and pulse mass were similar in HR and LR (Figure 1). There were no significant differences in the Approximate Entropy value, but the mode was lower ($P<0.05$) in HR, which suggests that there was a shorter latency between the onset of a pulse and the peak of the pulse episode (Figure 1). In addition, the AUC in the first hour of sampling (09:00-10:00h) was greater ($P<0.01$) in HR compared to LR. Thus, in the basal non-stressed state, HR had higher morning cortisol levels than LR (Figure 1 b and c).

Experiment 2a: Effect of insulin-induced hypoglycaemia on food intake, thermogenesis and plasma cortisol levels in LR and HR

Injection of insulin induced an equivalent degree of hypoglycaemia in LR and HR animals (Figure 2d). In response to this stressor, both plasma levels of cortisol ($P<0.0001$) and skeletal muscle heat production ($P<0.0001$) increased to a similar degree in LR and HR (Figure 2a and b). There was no effect of insulin-induced hypoglycaemia on food intake in either group (2c).

Experiment 2b: Effect of immune challenge on food intake, thermogenesis, plasma cortisol and inflammatory cytokine levels in LR and HR

Injection of LPS increased ($P<0.01$) plasma cortisol levels in LR and HR with a greater ($P<0.05$) effect in HR than in LR (Figure 3a). This divergence in cortisol response was

concomitant with different metabolic responses. Skeletal muscle thermogenesis was increased after LPS injection in both groups, but LR had higher muscle temperature responses than HR (Figure 3b). Similarly, LPS-treatment reduced food intake in both groups, but a greater degree in LR than in HR (Figure 3c). Treatment with LPS increased ($P<0.0001$) the secretion of TNFα, IL6 and IL10, to a similar degree in LR and HR. There was no effect of LPS treatment on the plasma levels of IL4 or IL12 in either group (Figure 3).

Experiment 2c: Effect of psychosocial stress on food intake, thermogenesis, plasma cortisol and catecholamine levels in LR and HR

Exposure to a barking dog increased ($P<0.05$) plasma cortisol levels in LR and HR with the response being greater ($P<0.05$) in HR (Figure 4a). In spite of this, skeletal muscle temperature was similar in LR and HR across the experimental period (Figure 4b). The psychosocial stress reduced ($P<0.05$) food intake in LR only (Figure 4c). Catecholamine response to barking dog was quantified by assessing the AUC for adrenaline, noradrenaline and DHPG (Figure 4d-f). The AUC for noradrenaline and DHPG were similar in LR and HR, but the adrenaline response was greater ($P<0.05$) in LR. This demonstrates an inverse relationship between plasma levels in cortisol and adrenaline in LR and HR animals in response to psychosocial stress.

Experiment 3a: Behavioral responses in the open field test

There was little difference in the behavioral response to isolation in LR and HR animals subjected to the open field test (Figure 5). The number of bleats, gate knocking and tunneling was similar in LR and HR. On the other hand, LR animals showed greater levels of activity whereby the total activity ($P<0.05$) and locomotor activity ($P<0.05$) were higher in LR compared to HR.

Experiment 3b: Behavioral response to the arena test

LR animals displayed less fear than HR (Figure 5b). LR animals spent a greater ($P<0.05$) amount of time in Zone 1 than did HR, indicating less fear to the human. On the other hand, tunneling behavior was exclusively observed in HR, suggesting that these animals have greater passivity compared to LR.

Experiment 3c: Food competition and proactive behavior test.

LR animals had a lower latency ($P<0.05$) to initiate feeding compared to the control group (Figure 5c). This suggests that the LR animals display a proactive coping strategy compared to HR.

Discussion

We have characterized cortisol secretion, metabolic indices and behavior in response to stress in animals selected for either high or low cortisol response to ACTH. HR animals had higher morning cortisol levels in plasma. In response to both immune and psychosocial stressors, secretion of cortisol was greater in HR than LR. Notably, this divergence in cortisol secretion was associated with differences in food intake, such that LR animals showed a greater reduction in food intake in response to stress than HR. In response to LPS, muscle temperature was increased to a greater degree in LR, suggesting that they develop an enhanced catabolic state in response to stress compared to HR. In addition to the metabolic differences in response to stress, LR and HR animals displayed diverse and disparate behavioral phenotypes. LR animals showed increased physical activity, relative fearlessness and a proactive coping style, whereas HR animals displayed a cohesive set of reactive behaviors. This suggests that differences in the propensity to obesity in HR and LR manifest due to a complex interplay between the control of food intake, energy expenditure, stress responsiveness and behavioral temperament. Overall, HR animals have increased propensity to obesity, which is driven by reduced energy expenditure (thermogenesis and physical activity), but also a tendency to have increased food intake in response to stressful stimuli when compared to LR.

Reports of effects of stress and elevated glucocorticoid concentrations on food intake have been dichotomous with a number of studies reporting conflicting findings. The comfort food hypothesis dictates that stress increases intake of foods high in fat and sugar (Pecoraro, Reyes et al. 2004, Dallman, Pecoraro et al. 2005). Increased intake of fat and sugar constitutes a negative feedback pathway, which

dampens the HPA axis. Further to this, clinical studies have shown cortisol responsiveness to be an important determinant of the effect of stress on food intake, whereby patients characterized as high cortisol responders tend to eat more after a stressful episode than those characterized as low cortisol responders (Epel, Lapidus et al. 2001, Tomiyama, Dallman et al. 2011, Groesz, McCoy et al. 2012, Tomiyama, Schamarek et al. 2012). Indeed recent work has shown that consumption of "comfort foods" is also associated with cortisol responses in that high cortisol responding patients are more likely to consume high fat/ high sugar foods after a stressful episode (Laugero, Gomez et al. 2002, Pecoraro, Reyes et al. 2004, la Fleur, Houshyar et al. 2005, Tomiyama, Dallman et al. 2011). Importantly, with respect to the present work, sheep are ruminants and do not derive either fat or sugar from their diet. Our sheep are maintained on a homogeneous diet of Lucerne chaff, deriving volatile fatty acids from their food. Thus, the nexus between the HPA axis, cortisol responsiveness and metabolic function extends to animals that are not mono-gastric.

The current study demonstrates that the effect of stress on food intake is determined not only by cortisol responsiveness but also by the type of stressor. We demonstrated that although an immune challenge (LPS) and psychosocial stress (barking dog) reduce food intake, there was no effect of a metabolic insult (insulin-induced hypoglycaemia) on feeding. Again, this may be a particular characteristic of the ruminant animal. Nonetheless, we demonstrate that LR animals are more affected by stress in terms of food intake whereby LPS reduced intake in both LR and HR animals, but food intake was lower in LR. Likewise, psychosocial stress reduced food intake in LR only. This would contribute to the maintenance of a lean phenotype in LR, consistent with our earlier work (Lee, Clarke et al. 2014).

Our previous work also demonstrated that LR and HR exhibit innate differences in the propensity to obesity (Lee, Clarke et al. 2014) when fed a high energy diet and that this is related to their inherent differences in thermogenesis in skeletal muscle. HR animals have a lower thermogenic output from skeletal muscle (Lee, Clarke et al. 2014) in response to feeding and to the central infusion of leptin. Interestingly, in

the non-stressed state, food intake is similar in LR and HR, which is concordant with results in humans, where patients characterized as having high and low cortisol responses had similar food intake at baseline, but food intake diverged after stress - high cortisol responders eat more than low cortisol responders (Epel, Lapidus et al. 2001, Tomiyama, Dallman et al. 2011). The present work shows that, after immune challenge, HR and LR show differences in food intake and skeletal muscle heat production, which reflects a greater negative energy balance in LR. Injection of LPS reduced food intake in HR and LR, but food intake was lower in the latter. In concert with this greater reduction in food intake, skeletal muscle heat production was elevated in LR, all leading to greater negative energy balance. Despite metabolic differences, circulating levels of cytokines were similar in LR and HR, suggesting that cortisol responsiveness did not impact on the immune response to LPS. LPS-treatment increased the circulating levels of some pro-inflammatory (TNFα and IL6) and anti-inflammatory (IL10) cytokines. On the other hand, plasma levels of IL4 and IL12 were not altered by LPS-injection. Thus, our cytokine data suggest that the different metabolic responses to LPS challenge in HR and LR are not driven by differences in immune function or altered sickness predisposition. Nonetheless in response to immune challenge LR animals are more likely to lose weight due to an enhanced 'catabolic' state. On the other hand, HR animals appear to protect their body weight and this aligns with the previously observed increased propensity to obesity (Lee, Clarke et al. 2014). This supports the notion that cortisol responsiveness is a marker for innate differences in metabolic set-point and altered susceptibility to obesity (Knott, Cummins et al. 2008, Block, He et al. 2009, Lee, Clarke et al. 2014).

In addition to immune challenge, we demonstrated divergence in cortisol response, food intake and plasma adrenaline levels after psychosocial stress in LR and HR. LR animals display an attenuated increase in cortisol, but an inverse adrenaline response whereby the increase in plasma adrenaline is heightened in LR compared to HR. Stress increased noradrenaline and the metabolite DHPG to an equivalent degree in LR and HR. The difference in cortisol secretion in response to barking dog corresponded to differences in food intake, as outline above, in that psychosocial

stress reduced food intake in LR only. Interestingly, we demonstrated an inverse relationship between the catecholamine, adrenaline, and cortisol responses to stress. This inverse relationship is characteristic of the neuroendocrine differences between proactive and reactive coping styles (Koolhaas, Korte et al. 1999), which is discussed in detail below.

Interestingly, in contrast to psychosocial and immune challenges, the cortisol responses to insulin-induced hypoglycaemia did not differ between HR and LR. This metabolic challenge stimulated the HPA axis, but the effect was equivalent in HR and LR animals. This contrasts previous work in rams whereby insulin-induced hypoglycaemia differed between high and low cortisol responders, similar to an ACTH challenge (Knott, Cummins et al. 2010). Earlier work in sheep has also shown that males are more susceptible to the effects of insulin-induced hypoglycaemia than females, irrespective of gonadal steroids (Turner, Canny et al. 2002). This previous study demonstrated that an insulin challenge increases cortisol levels to a greater degree in males than in females (Turner, Canny et al. 2002). Such sexual dimorphism may explain why there are differences between the two studies in different sexes. Although Knott and colleagues (Knott, Cummins et al. 2010) measured cortisol responsiveness it would be interesting to know whether the observed differences in cortisol levels correlated to acute change in food intake or thermogenic output in the rams. Nonetheless, the current study demonstrates that in female sheep insulin-induced hypoglycaemia stimulates cortisol secretion and increases muscle temperature, without an associated effect on food intake. Furthermore, we demonstrated that these effects are similar in ewes that have been selected for either high or low cortisol responsiveness via an ACTH challenge.

Further to the metabolic sequelae in response to stress, we demonstrated divergence in behavior, temperament and coping strategy in LR and HR. In obese patients, emotional eating is positively associated with impulsiveness and depression, whereas restrained eating correlates with openness, conscientiousness and extraverted personality traits (Elfhag and Morey 2008). Moreover, successful weight loss is less likely in obese subjects that exhibit increased novelty seeking

behavior (Sullivan, Cloninger et al. 2007), which suggests a lack of impulse control. We therefore sought to identify possible behavioral correlates in LR and HR that may contribute to altered propensity to obesity. Indeed, in humans and a number of animal models, differences in cortisol responsiveness have been shown to associate with differences in behavior, temperament and coping strategies. In humans, low cortisol response to stress is associated with higher neuroticism in women, low extraversion in men and low openness in both sexes (Oswald, Zandi et al. 2006). Thus it is possible that differing set-point of the HPA axis is associated with personality traits that are also evident in obese subjects.

Various animal models have demonstrated that low cortisol responses are associated with increased aggression (Touma, Bunck et al. 2008, Murani, Ponsuksili et al. 2010, Terenina, Babigumira et al. 2013). In pigs, recent studies have identified a number of single nucleotide polymorphisms that associated with both HPA axis activity and aggression. Indeed, these molecular variants are largely found in genes that govern the HPA axis, catecholaminergic and serotonergic systems (Terenina, Babigumira et al. 2013). In the current study we link low cortisol response to reduced fearfulness, whereby LR animals spent a greater amount of time in zone 1 (closest to the human) compared to HR. In addition to aggression, innate differences in cortisol responsiveness correlated to differences in coping strategy. A coping strategy refers to a cohesive set of behaviors and physiological responses to stress. In this regard, LR animals displayed a proactive coping strategy, because they have a low cortisol response but a heightened sympathetic response to psychosocial (barking dog) stress - this is a neuroendocrine hallmark of a proactive coping strategy (Koolhaas, Korte et al. 1999). Furthermore, the LR animals showed a shorter latency to the onset of feeding in the food competition assay and increased physical activity in the open field test, supporting the proposition that LR animals have a proactive phenotype. As to whether a proactive coping strategy leads to altered susceptibility to obesity or to weight loss remains unknown, but it has been hypothesized that animals of a proactive nature are more likely to expend energy - the proactive behaviors such as aggression and physical activity are more likely to expend energy than the reactive behaviors such as freezing (Garland, Schutz et al. 2011). We have

demonstrated that LR increase physical activity in response to stress (as shown by isolation in the open field), which may contribute to their relative resistance to weight gain.

In conclusion, we demonstrated that cortisol responses to ACTH can be used to predict the metabolic and behavioral responses to stress. We show that animals characterized as LR have reduced cortisol secretion in response to LPS and psychosocial stressors that coincide with a greater reduction in food intake. Furthermore, immune challenge causes a greater increase in skeletal muscle thermogenesis in LR than HR. Despite the metabolic differences, HR and LR had similar levels of circulating cytokines, suggesting that immune function is similar in the two groups. In addition to the metabolic response to stress, we demonstrate that LR animals adopt a proactive coping strategy, which may also contribute to an innate increase in energy expenditure. Thus, we demonstrate that in response to stress animals characterized as LR are more likely to enter negative energy balance and are therefore relatively protected against diet-induced obesity. On the other hand, HR are more likely to defend their body weight under stressful conditions and are therefore more susceptible to diet-induced obesity. Behavioural testing may identify individuals that are susceptible to obesity.

Acknowledgements

The authors wish to acknowledge Prof Johannes Veldhuis who conducted the deconvolution analyzes on the baseline cortisol data. We also wish to thank Mr Bruce Doughton, Ms Lynda Morrish and Ms Elaine Chase for animal husbandry and technical assistance and Ms Sofie Selah for assistance with the cortisol assays. This work was supported by the National Health and Medical Research Council project grant #1005935 (B.A.H).

Figure legends

Figure 1. Cortisol secretory profiles in animals selected for low (LR) and high (HR) cortisol responses after synacthen challenge. The cortisol response to adrenocorticotropin (ACTH) challenge was greater in HR compared to LR (Panel A). Characterization of baseline cortisol levels in LR (white symbols) and HR (black symbols) is shown in panels B-D. Plasma levels of cortisol were elevated during the first hour of sampling (0900-1000h), but there was little difference in concentrations across the remaining sampling period (Panel B). We further analyzed the morning plasma cortisol levels by measuring the area under the curve (AUC: 0900-1000h, Panel C). The AUC for cortisol was higher in HR compared to LR. Deconvolution analyzis of the baseline cortisol data is presented in Panel D. Secretory parameters including the number of pulses, basal secretion, pulsatile secretion, total secretion, pulse mass and the approximate entropy score were similar in LR and HR, whereas the mode was higher ($P<0.05$) in LR, which is consistent with an elevation in cortisol in HR compared to LR. All data are presented as the mean±SEM, n=7-10/group. *$P<0.05$, **$P<0.01$ LR compared to HR.

Figure 2. Effects of insulin-induced hypoglycaemia on plasma levels of cortisol and glucose, temperature in skeletal muscle and food intake in ewes selected for low (LR: white symbols) and high (HR: black symbols) cortisol responsiveness. Insulin-induced hypoglycaemia was induced by a single injection of actrapid at 1200h, as dictated by the arrow. Hypoglycaemia increased plasma cortisol levels (Panel A) as well as skeletal muscle temperature (Panel B) ($P<0.0001$ effect of time) in both LR and HR. There was no significant effect of insulin-induced hypoglycaemia on food intake in either group (Panel C), nor were there any differences in plasma cortisol levels or muscle temperature in LR and HR. Injection of actrapid caused an equivalent hypoglycaemia in LR and HR (Panel D). All data are presented as the mean±SEM, n=5/group.

Figure 3. Effects of lipopolysaccharide (LPS) treatment on plasma levels of cortisol and cytokines, temperature in skeletal muscle and food intake in ewes selected for low (LR: white symbols) and high (HR: black symbols) cortisol responsiveness.

Injection of LPS (as shown by the arrow at 1200h) increased plasma cortisol in both LR and HR ($P<0.0001$ effect of time), however the increase in cortisol was greater in HR (Panel A). LPS treatment also increased skeletal muscle heat production in both groups ($P<0.0001$, effect of time) but the temperature was higher ($P<0.05$) in LR (Panel B). LPS treatment also reduced food intake in both LR ($P<0.01$) and HR ($P<0.05$) compared to control (baseline) food intake (Panel C). The reduction in food intake, however, was greater ($P<0.05$) in LR compared to HR. In addition we measured plasma levels of interleukin 4 (IL4, Panel D), interleukin 6 (IL6, Panel E), interleukin 10 (IL10, Panel 10), interleukin 12 (IL12, Paenl G) and tumor necrosis factor α (TNFα, Paenl H). Plasma cytokine levels were similar in LR and HR before and after injection of LPS. Injection of LPS increased plasma levels of IL6, IL10 and TNFα ($P<0.0001$ effect of time), but there was no effect on plasma levels of IL4 or IL12. All data are presented as the mean±SEM, n=5/group. *$P<0.05$ LR compared to HR, $^{a}P<0.05$, $^{aa}P<0.01$ compared to control, $^{b}P<0.05$ LR compared to HR.

Figure 4. Effect of psychosocial stress on plasma levels of cortisol and catecholamines as well as metabolic indices in low (LR: white symbols) and high (HR: black symbols) cortisol responding animals. Plasma cortisol concentration was increased in LR and HR in response to barking dog stress, but this effect was greater in HR (Panel A). There was little effect of psychosocial stress on skeletal muscle temperature in either LR or HR (Panel B), whereas food intake was decreased in response to stress in LR only (Panel C). With regards to plasma catecholamine levels, the adrenaline response to stress as determined by measuring the area under the curve (AUC) was greater in LR compared to HR. Plasma noradrenaline and it's metabolite, dihydroxyphenylglycol (DHPG) was similar in LR and HR. The arrow depicts the onset of the barking dog stress. All data are presented as the mean±SEM, n=5/group. *$P<0.05$ LR compared to HR.

Figure 5. Behavioral differences in animals selected for either low (LR: white bars) or high (HR: black/ grey bars) cortisol response to adrenocorticotropin. Behavioral responses were characterized in the open field test (Panel A), arena test (Panel B) and a food competition assay (Panel C). LR animals had increased locomotion and

total physical activity in response to isolation in the open field test. Furthermore, LR animals displayed reduced fearfulness in the arena test as demonstrated by a greater amount of time spent in Zone 1, closest to the human. On the other hand, tunneling which is a behavior associated with active avoidance was only present in HR. Finally, the LR animals showed a proactive coping style which was associated with the aforementioned behaviors as well as a reduced latency to feed when compared to control animals in the food competition test. All data are presented as the mean±SEM, n=5/group. *P<0.05 LR compared to HR.

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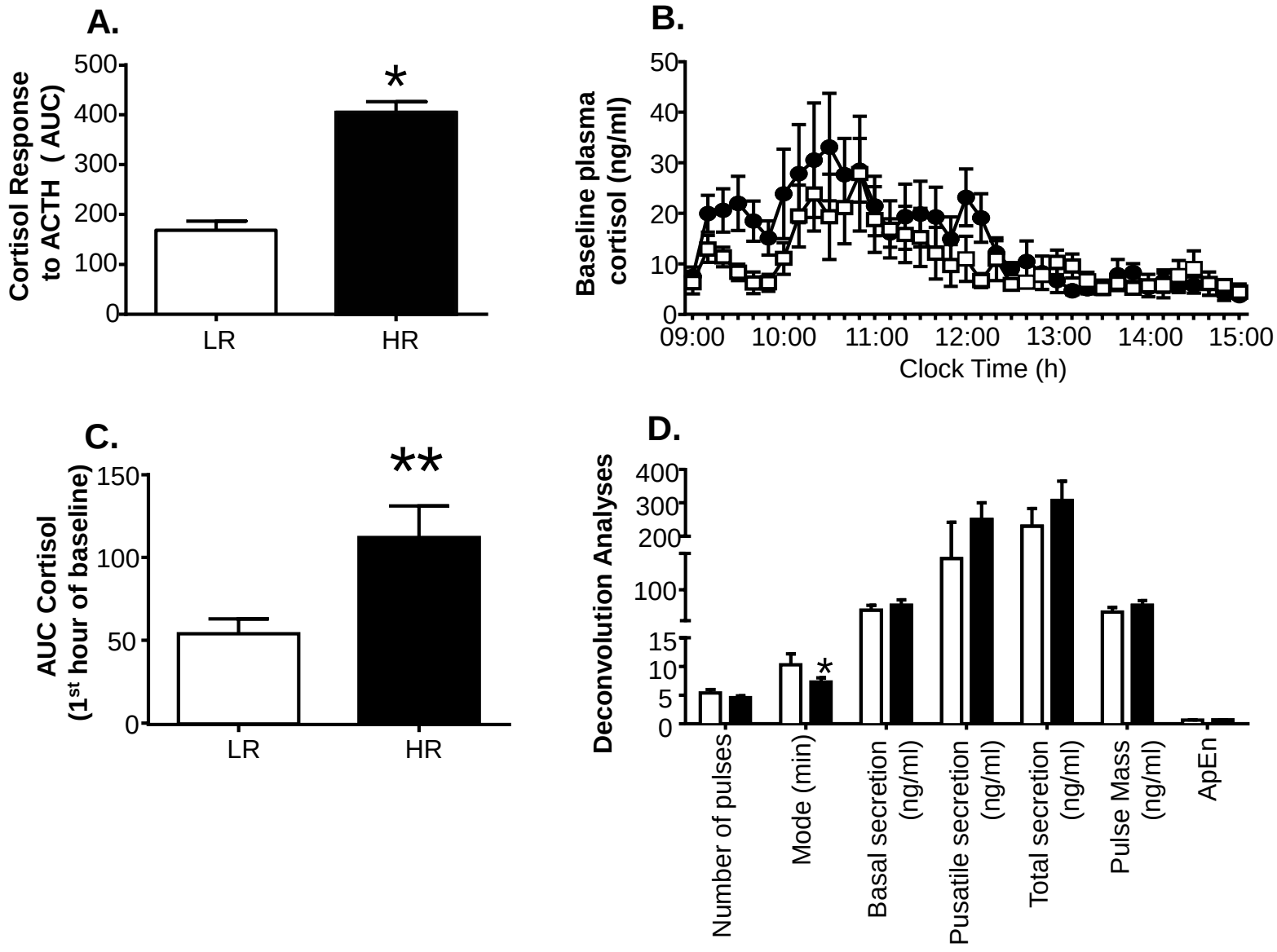

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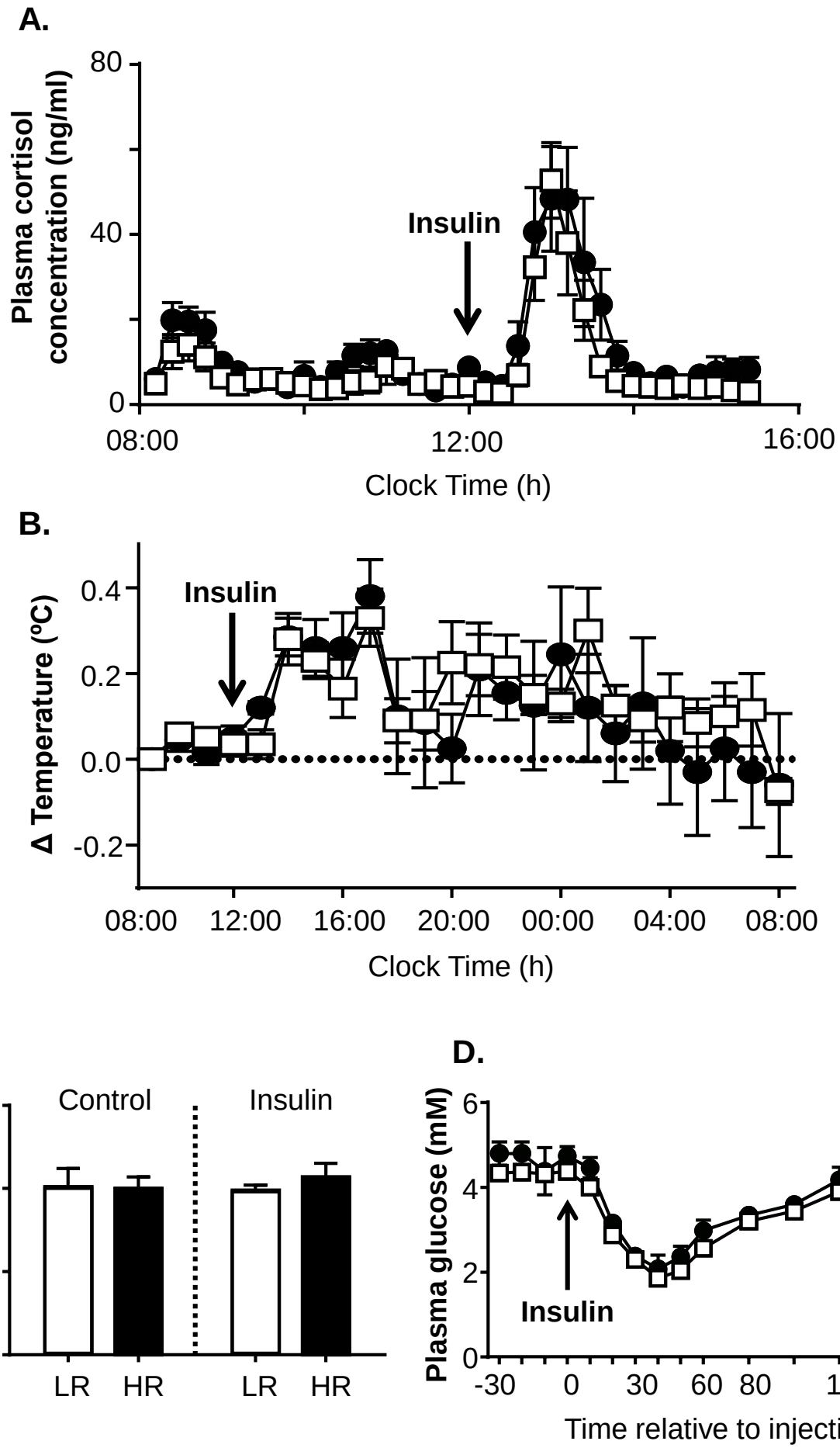

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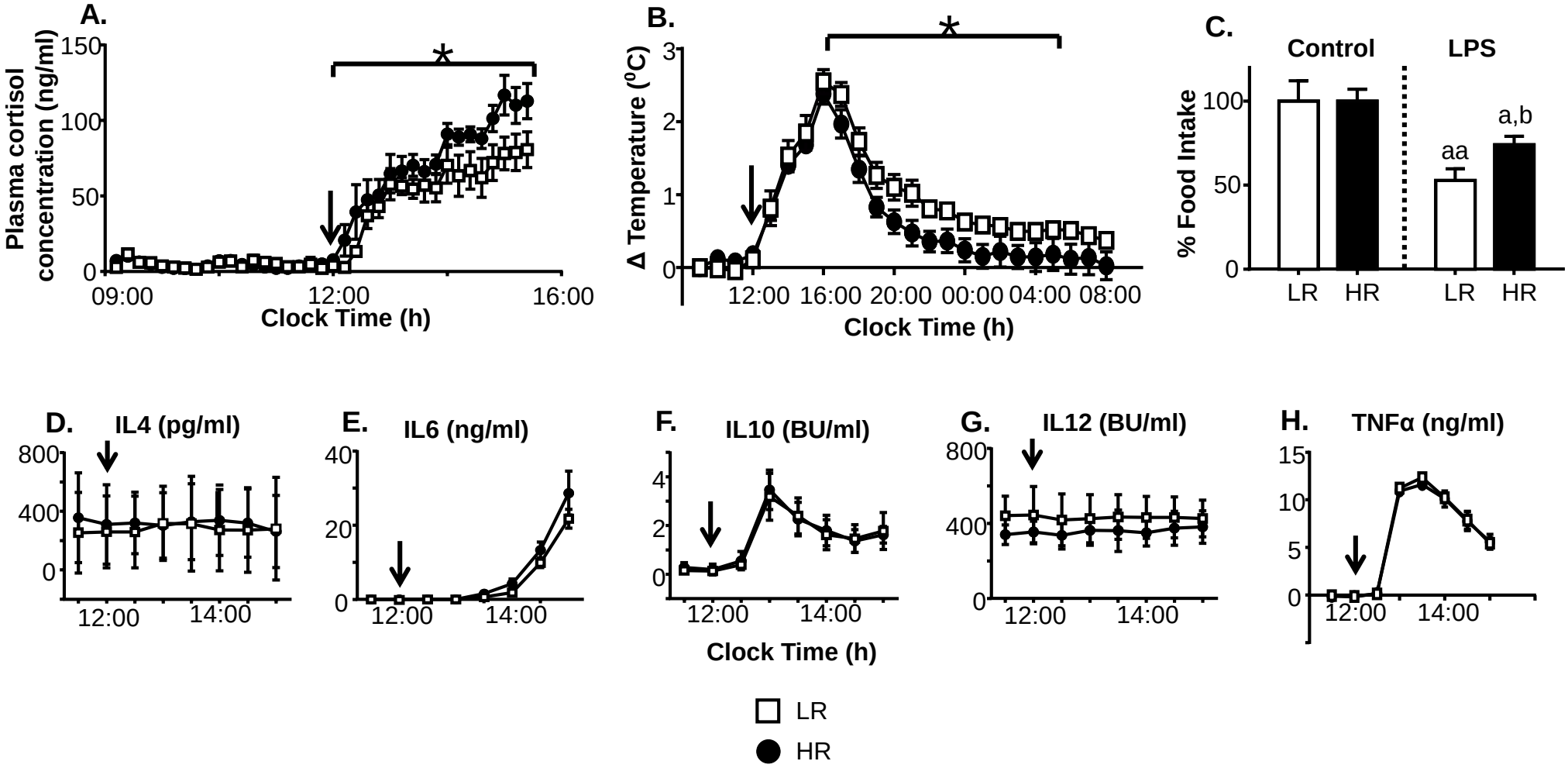

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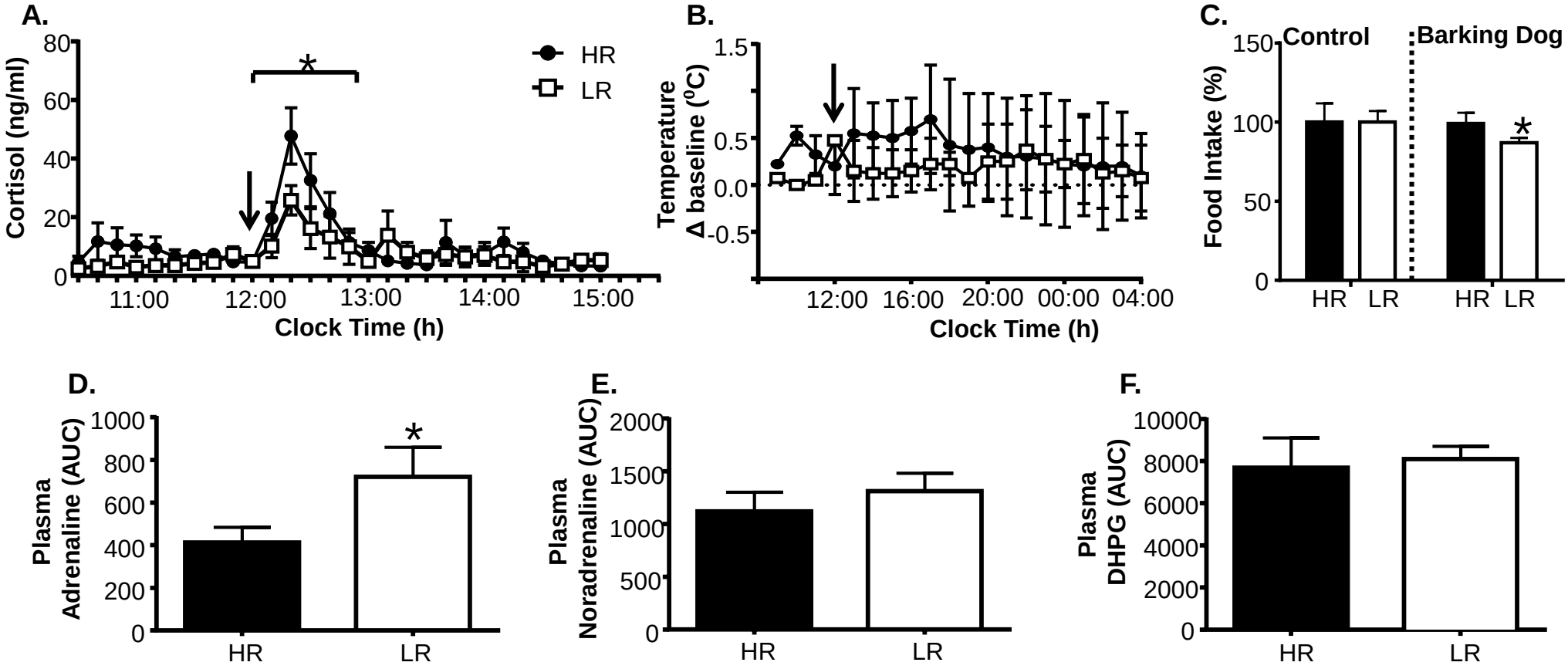

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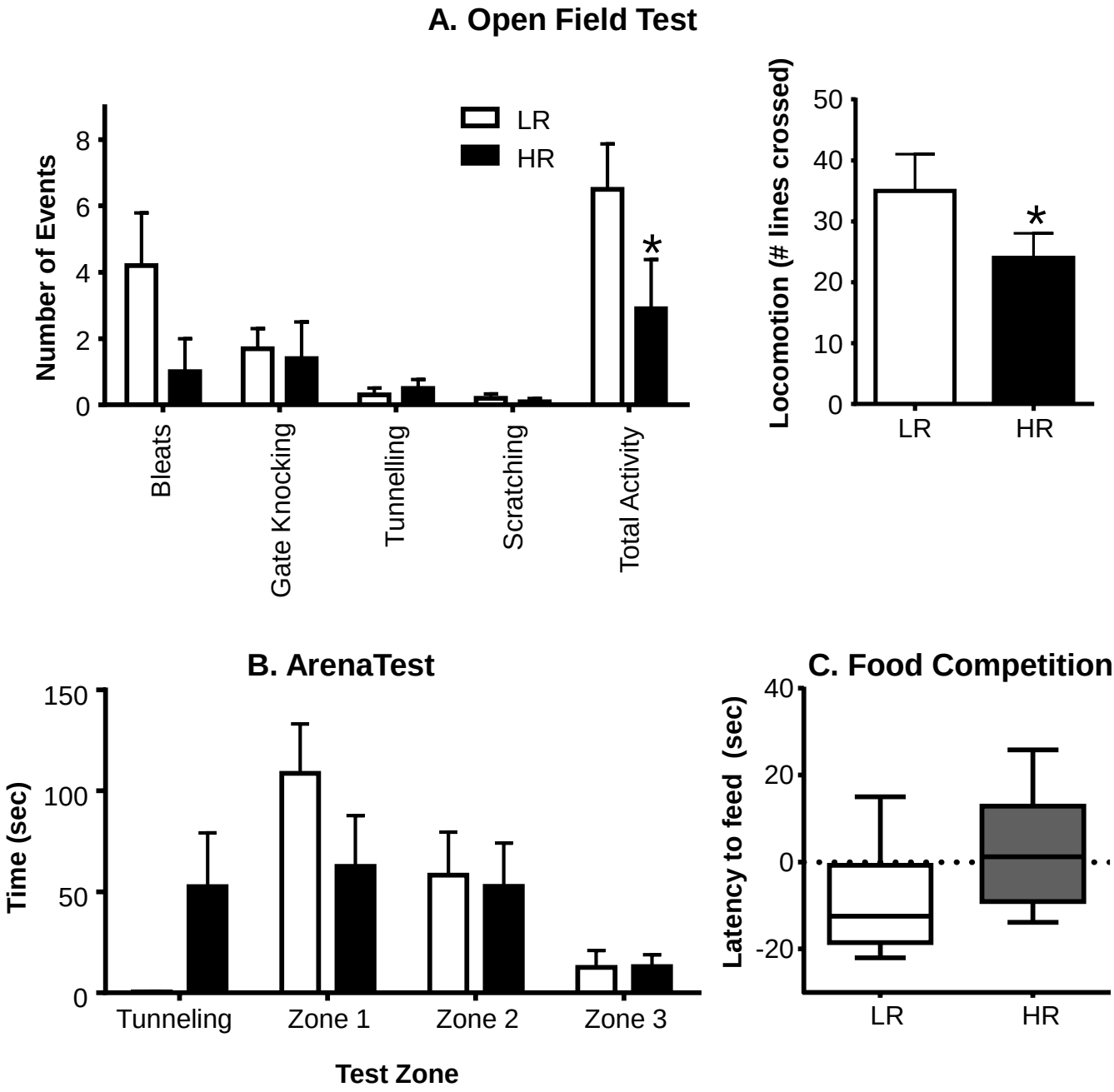

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