Stress responses in an outbred, wild-derived hamster, *Phodopus campbelli*: Individuality in the cortisol response to acute restraint stress and the absence of strong association with behavioural responses to acute restraint

by

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Abstract

Glucocorticoid reactivity is often used as a biomarker of emotional stress status in animals and man. Likewise, behavioural responses of individuals to standardized stresses are also widely used to assess the magnitude of perceived stress. Unfortunately, there is little evidence that widely used physiological measures and widely used behavioural measures yield similar, or even correlated, results. The current study provides an explicit test of the relationship between the two approaches.

Dwarf hamsters (*Phodopus campbelli*) were subjected to a standardized, on-back restraint in a socially subordinate position and had plasma samples drawn under brief, home-cage, isoflurane anesthesia to quantify cortisol before and after the acute stress. Individual responses were transformed into parameters describing the absolute change in cortisol, the proportional changes in cortisol, and the overall exposure to cortisol across stress and recovery. There was exceptional individual variability in cortisol responses to the standardized stressor, including monotonic declines, and cluster analysis failed to effectively partition between-individual variation by sex, age, or housing conditions, for any parameter.

Next, physiological measures, and a battery of behavioural tests routinely used to assess, anxiety, stress, and emotionality differences, as well as resistance to capture and intruder aggression, were combined in an expanded population including individuals from genetic lines selected to increase or decrease cortisol concentration. In general, dwarf hamsters showed less neophobia than is typical of laboratory mice and rats (e.g. animals quickly dropped from the elevated plus maze). In addition, results were

combined to create behavioural indices by orienting each test score relative to an emotionality continuum and combining tests based on, *a priori*, expectations of similarity. Multivariate regression failed to detect covariation between physiological and behavioural measures, despite the large number of parameters describing those responses.

Although the experimental design did not include replication, or comparison of physiological responses to different stressors, it was clear that behavioural and physiological responses were neither interchangeable nor statistically associated. Thus, results challenge widespread assumptions about the interaction between the glucocorticoid response to stress, and the manifestation of that stress through alterations in behaviour. Additional research exploring physiology-behaviour associations in genetically diverse populations is warranted.

Co-Authorship

The results presented in Chapter 3 are a joint effort of F.S. Guimont and K.E. Wynne-Edwards. The manuscript was published under the following reference: Guimont, F.S., Wynne-Edwards, K.E., 2006. Individual variation in cortisol responses to acute 'on-back' restraint in an outbred hamster. Hormones and Behavior 50, 252-260. Chapter 4 is also another joint effort of F.S. Guimont and K.E. Wynne-Edwards and the manuscript has been prepared for submission. All the data analysis and writing of this thesis was conducted by F.S. Guimont and K.E. Wynne-Edwards.

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11βHSD	11β- hydroxysteroid dehydrogenase
5-HT	5-hydroxytryptamine or serotonin
ACTH	adrenocorticotropic hormone
AUC	area under the curve
C	control population
CRF	corticotrophin-releasing factor or CRH
CRH	corticotrophin-releasing hormone or CRF
EPM	elevated plus-maze
GABA	gamma-aminobutyric acid
GH	growth hormone
GR	glucocorticoid receptor
H	high cortisol amplitude population
HB	holeboard
HPA	hypothalamic-pituitary-adrenal
IT	intruder test
L	low cortisol amplitude population
L/D	light/dark box
OF	open-field
PCA	principal component analyses
PVN	paraventricular nucleus

Chapter 1
General Introduction

Natural selection maximizes the reproductive success of the best-adapted individuals even at the expense of their health and longevity (Nesse, 1999). Thus, individual characteristics of coping with stress are an important part of Darwinian fitness (Korte et al., 2005). As stress can be damaging for individual health if encountered with too much intensity or frequency (Moberg, 2000), different situations or environments will favor certain types of stress responses more than others (Sih et al., 2004). Thus, coping styles can be evolutionarily stable strategies that persist in a population (Koolhass et al., 1999; Korte et al., 2005; Koolhaas, 2008). For example, aggressive individuals or '*hawks*' and cooperative and relatively passive ones or '*doves*' can have differential fitness advantages (Korte et al., 2005).

In an environment with concentrated resources, *hawk*-type individuals have a fitness advantage by their aggressive behavior over the *dove*-type. On the other hand, when resources are scarce, the *doves* have the advantage since the *hawks* will spend too much energy to get the dispersed resources. Accordingly, *hawks* and *doves* have different HPA (Hypothalamic-Pituitary-Adrenal) axis output. *Hawks* have a high baseline concentration and a relatively low increase in glucocorticoid concentration and are often terms 'proactive whereas *doves* have a lower baseline concentration and a more dramatic increase in response to stress, making them 'reactive' (Korte et al., 2005). Of course, this does not mean that one individual will always react in a strictly proactive or reactive manner. Coping style can change within an individual over time or in response to other biologically relevant variables such as social status.

To date, studies of the stress response have tended to emphasize either the molecular and physiological aspects of the response, or the behavioural response (Koolhaas et al., 2007; McEwen, 2007). However, there is little evidence supporting the equivalence or differences

between approaches. On the physiological level, measurements like glucocorticoid change are not always indicative of a variation in stress level (Mormède et al., 2007). As well, behavioural measurements of stress responses seem to be highly dependent on the ecological context (Rushen, 2000).

In addition, studies that examine multiple measures within known individuals are largely restricted to inbred laboratory rodents and humans (Williams, 2008). Inbred strains are, by definition, limited in their ability to explore population variation between individuals (Øverli et al., 2007; Touma et al., 2008). Human studies are challenging to interpret because it is difficult to separate the stress from the interaction between the researcher and the subject. On the other hand, human subjects can communicate their reactions to a situation, and inbred laboratory rodents have well-established batteries of behavioural tests that are widely used to evaluate emotion-related stress (Rushen, 2000).

The current thesis develops a new model system that is expected to make a novel contribution to our understanding of individual variability, and the associations between behavioural and physiological measures of the stress response. The model species is the Djungarian dwarf hamster (*Phodopus campbelli*), an outbred, wild-derived, small-bodied rodent that is amenable to rearing under standard laboratory conditions.

Chapter 2

Literature Review

The notion of 'stress' was developed from the studies of Hans Selye (1907-1982). Selye (1936) first described stress as the 'syndrome of just feeling sick'. He also observed that the chronic inability to cope with stress resulted in increased in adrenal weight, decreased thymus weight, and the formation of gastric ulcers. Over the years, evidence pointed to psychological adversity as the most potent stressor (de Kloet et al., 2008; Koolhass, 2008). However, the term 'stress' has been used very broadly in biology. Unlike most diseases, stress has no defined etiology or prognosis (Moberg, 2000). Nevertheless, 'stress' can be generally defined from a biological point of view as 'the biological response elicited when an individual perceives a threat to its homeostasis' (Moberg, 2000). Thus, keeping stress from threatening the homeostasis of the body is an essential adaptation for survival.

2.1 Stress response and the HPA axis

When animals are exposed to actual or potentially dangerous situations, an elaborate response is orchestrated by the brain. Activation of the hypothalamic-pituitary-adrenal (HPA) axis, with the subsequent release of adrenocorticotropic hormone (ACTH) and glucocorticoids into the blood is one of the prototypical responses to all stressful situations. Thus, changes in plasma concentration of glucocorticoids have been consistently found to be associated with such situations and in the majority of stress studies, the HPA axis has been the primary neuroendocrine axis monitored (Moberg, 2000; Gagliano et al., 2008). Since animals perceiving a stressor are likely to increase their level of circulating glucocorticoids produced through their HPA axis, capture, handling, restraint or immobilization can be used as a standard acute stressor (Armario, 2006). The plasma glucocorticoid response to such a stressor is typically a rise in concentration that starts several minutes after the stress onset and peaks between 15 and 20 minutes later

(García et al., 2000). In domesticated chickens and Japanese quail, glucocorticoids then decline whereas in most species of free-living birds that have been studied, corticosterone levels are still elevated 60 minutes after capture (Cockrem et al., 2008). In inbred laboratory rodents, the stress response varies between strains, but glucocorticoid concentration generally tends to go down after the stressor has ended (Grota et al, 1997; Gómez et al., 1998). However, convincing correlations between HPA axis activity and coping style or other anxiety-related behavior are not always found and seem to depend on the type of stressor used (Koolhaas et al., 2007; Gagliano et al., 2008) as well as the method used to quantify it (Pruessner et al., 2003; Lee et al., 2007).

2.2 HPA axis physiology

The HPA axis is basically a neuroendocrine system that links the central nervous system and the adrenal glands (Figure 1). More specifically, the hypothalamus controls the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which, in turn, stimulates the secretion of glucocorticoid hormones by the adrenal cortex. The main hormone of the HPA axis varies among species; cortisol is predominantly secreted in humans, cattle, sheep, pigs, mink, foxes, fish and hamsters, as opposed to the dominant secretion of corticosterone in birds, rats and mice (Mormède et al., 2007). These hormones, known collectively as glucocorticoids, are cholesterol-derived steroids synthesized in the fascicular zone of the adrenal cortex under the control of ACTH.

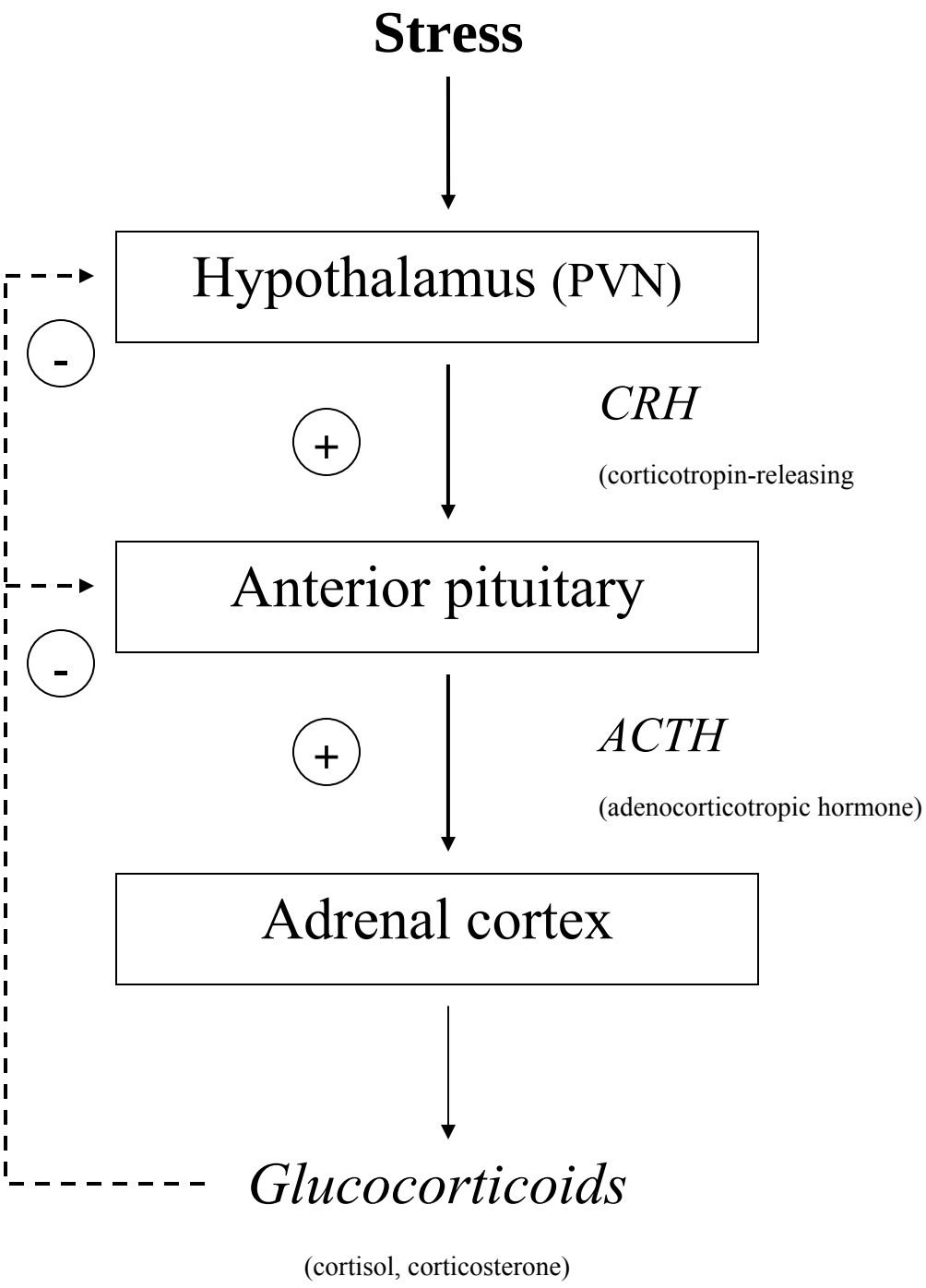
Stress
Hypothalamus (PVN)
-
+
CRH
(corticotropin-releasing
Anterior pituitary
-
+
ACTH
(adenocorticotropic hormone)
Adrenal cortex
Glucocorticoids
(cortisol, corticosterone)

Figure 1. Schematic diagram representing acute stress reaction mediated by the hypothalamic-pituitary-adrenal (HPA) axis. The perception of a stressful stimulus (external or internal) initiates a response by neurosecretory neurons of the paraventricular nucleus (PVN) in the hypothalamus, which produces corticotrophin-releasing factor hormone (CRH). The CRH released into the hypophysial-portal circulation stimulates secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. ACTH then promotes synthesis (represented by the black line), and release, of glucocorticoids into the systemic circulation by the adrenal cortex. The subsequent rise in plasma glucocorticoid concentration stimulates central mineralocorticoid receptors, which reduce the release of CRH, and thereby reduce further synthesis and release of glucocorticoids from the adrenals (negative feedback represented by the dashed line).

The ACTH is produced by corticotrophs, which are specialized cells of the anterior pituitary gland (Miller and O'Callaghan, 2002). Its release is triggered by the coordinated action of two neuropeptides, corticotropin-releasing hormone (CRH) and vasopressin (AVP). Both neuropeptides are synthesized in specialized neurons of the paraventricular nucleus (PVN) of the hypothalamus and released in the capillary bed of the median eminence, from where they reach the pituitary directly via the hypothalamic–pituitary portal circulation. The PVN receives numerous inputs from other hypothalamic nuclei. These inputs carry metabolic and circadian signals from the brain stem, the subfornical organ, and the limbic system. The brain stem relays neural inputs from the periphery while the subfornical organ monitors blood plasma composition. Finally, the limbic system generates signals related to the emotional state (Engelmann et al., 2004).

This multiplicity of signals converging in the PVN explains the sensitivity of the HPA axis to a wide range of stimuli from both internal and external origins. Furthermore, glucocorticoids exert a negative feedback on the HPA axis by acting on the pituitary corticotrophs, the PVN, and higher levels in the central nervous system (Herman et al., 2005). This feedback action of glucocorticoids participates in the return of HPA axis activity to basal levels after stimulation (Manteuffel, 2002). Most circulating glucocorticoids (approx. 90%) are bound to proteins, principally albumin and corticosteroid binding globulin (CBG), a specialized glycoprotein that binds cortisol and corticosterone with high affinity and regulates its bioavailability (Gayard et al., 1996; Breuner and Orchinik, 2002). Due to its lipophilic structure, the free fraction of glucocorticoids can easily cross biological membranes, including the blood-brain barrier and cellular membranes, where they bind to receptors.

Glucocorticoids interact with mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) that, upon activation by their ligand, translocate to the cell nucleus to activate or inhibit gene expression by acting as transcription factors (Necela and Cidlowski, 2004; Pascual-Le Tallec and Lombes, 2005). The affinity of glucocorticoid for MR is about ten times higher than for GR (de Kloet et al., 2008). The MR affinity is high enough to maintain occupancy between the hourly glucocorticoid pulses, whereas GR binding tracks circulating glucocorticoid concentration changes produced by the ultradian rhythm and stress response (Conway-Campbell et al., 2007).

However MR is not strictly specific to glucocorticoids. In the peripheral circulation, aldosterone is the primary hormone activating the mineralocorticoid receptor. Aldosterone is released by the adrenal cortex under the influences of the renin-angiotensin system and ACTH. In the kidney, salivary glands and colon, (all tissues that are responsive to aldosterone and involved in water and electrolyte metabolism) the MRs are protected from glucocorticoid action by 11β-hydroxysteroid dehydrogenase (11βHSD), an enzyme that metabolizes cortisol into its inactive derivative, cortisone (Stewart and Krozowski, 1999). Detailed information on the metabolic effects of glucocorticoids, which are numerous and complex, can be found in Sapolsky and colleagues (2000).

Glucocorticoids have catabolic activity (both proteolytic and lipolytic), in peripheral tissues and anabolic activity in liver tissue (including gluconeogenesis and protein synthesis; McMahon et al., 1988). Since glucocorticoids also reduce the entrance of glucose into cells, they increase blood glucose and insulin. Furthermore, glucocorticoids increase food intake by an action in the brain so that the increase of energy availability is a coordinated process via peripheral and central mechanisms (Tempel and Leibowitz, 1994). When they are not used in the stress response, the combination of increased glucocorticoids and insulin leads to the storage of energy as fat in adipose tissue. The net effect is an increase of fat deposits at the expense of tissue

proteins (Devenport et al., 1989). Thus, the HPA axis is a dynamic system under the control of many variables that facilitates an adaptive response to restore physiological homeostasis (Slominski et al., 2007).

2.3 Individual variation of the HPA axis

Variation between individuals in HPA axis activity is well documented. Within individuals, however, there is evidence that the pattern is consistent in response to different stressful events (pigs, Hennessy et al., 1988; rats, Dellu et al., 1996; humans, Huizenga et al., 1998; birds, Love et al., 2004; Blas et al., 2007). Moreover, twin and family studies (Meikle et al., 1988; Linkowski et al., 1993; Inglis et al., 1999) have identified heritable components of these response patterns in humans. However, even though there appears to be intra-individual stability in glucocorticoid reactivity, large inter-individual variations have been described between inbred strains of mice (Levine and Treiman, 1964; Popova and Koryakina, 1981; Jones et al., 1998), rats (Castanon et al., 1993, 1994; Armario et al., 1995; Sarrieau et al., 1998; Sarrieau and Mormède, 1998), and among farm animal breeds (Garcia-Belenguer et al., 1996; Désautés et al., 1997; Hay and Mormède, 1998).

To investigate the genetic component of the glucocorticoid response to acute stressors, bird strains have been selected for divergent adrenal responses to ACTH (Edens and Siegel, 1975), immobilization (Satterlee and Johnson, 1988), cold (Brown and Nestor, 1973), restraint (Evans et al., 2005) or social stress (Gross and Siegel, 1985). In addition, salmonid fish strains have been selected for divergent responses to confinement stress (Fevolden et al., 1999; Pottinger and Carrick, 1999). Finally, mice had been also selected recently for divergent corticosterone response to restraint (Touma et al., 2008).

These data seem to point to a robust genetic component in the concentration and reactivity of glucocorticoid concentration in response to stress, leading to variation in the

behavioural reactions of those individuals (Solberg et al., 2006). However, a change in concentration is not the only component of the stress response that could vary, and could be heritable. Genetic factors could also influence the bioavailability of corticosteroid hormones. In pigs, polymorphisms of the CBG gene influence circulating cortisol levels (Désautés et al., 2002; Ousova et al., 2004; Geverink et al., 2006). In addition, large differences have been found in the efficiency of corticosteroid receptors (e.g.: DeRijk et al., 2002; Marissal-Arvy et al., 2004) as well as corticotropin-releasing hormone receptors (Tochigi et al., 2006; Kadarmideen and Janss, 2007).

Individual variation can also arise from environmental influences, including the prenatal and early postnatal environments. Sustained changes in HPA axis responsiveness have been found in pigs after prenatal restraint stress (Tuchscherer et al., 2002; Clinton et al., 2008), repeated exposures to noise (Otten et al., 2004; Kanitz et al., 2005), social isolation during early infancy (Kanitz et al., 2004; Tuchscherer et al., 2004) and neonatal handling (Weaver et al., 2000). Similar findings also occurred in wild birds (Blas et al., 2007; Love and Williams, 2008). Early influences have also been extensively studied in laboratory animals and influence emotional reactivity in adults, including neuroendocrine responses (Meaney et al., 1991).

Laboratory environments control the physical and social experiences of individuals, and can therefore be used to study responses to isolated selection pressures. However, the strains and species of mammals readily reared in laboratory situations are typically inbred, so that there is less raw individual variation than might be expected in a wild population.

2.4 Animal Model: Phodopus campbelli

The Djungarian dwarf hamster (*Phodopus campbelli*) is native to the arid semi-deserts of Central Asia, near the Altai Mountains (Wynne-Edwards, 2003). This harsh environment has shaped the physiology and the behavior of *P. campbelli*. *Phodopus* spp. have short limbs, digits, and tail, as

well as dense coat of hair covering the pads of the hind feet resulting from adaptations to a cold, dry seasonal habitat (Wynne-Edwards, 2003). Also, the ecological conditions in which *Phodopus* has evolved seem to have shaped their parental behavior. Constraints on maternal water balance and thermoregulation due to differential fitness in a desert environment were likely responsible for the evolution of biparental care in *P. campbelli*. Its sister species, *P. sungorus*, from which it diverged around one million year ago, does not express paternal care (Wynne-Edwards and Timonin, 2007) and is found in an environment where water stress and winter temperatures are less severe (Wynne-Edwards, 2003). This species easily made the transition to laboratory rearing, with every wild-caught individual breeding successfully over three importations in 1990-1998 (Wynne-Edwards, 2003). This wild-derived population has since been maintained at a population size of around 400 individuals with strict limitations on inbreeding. As such, ancestral differences between species in the genus, in circadian patterns of activity (Wynne-Edwards et al., 1999), and developmental responses to photoperiod (Timonin et al., 2006), are retained in this population and are supported by studies in the field (Wynne-Edwards et al., 1999). Thus, the laboratory population of *P. campbelli*, maintained since 1990 at Queen's University in Kingston, is a small-bodied rodent, with a different social structure than rats or mice, which is expected to have a larger population genetic diversity than other mammals previously studied for variability in the stress response.

Limited research on glucocorticoids in *Phodopus* has been conducted. The circadian cycle of cortisol secretion in male *P. campbelli* shows a nadir around 19h00 (Reburn and Wynne-Edwards, 2000), and 10 minutes following restraint stress both male and female *P. campbelli* show a significant increase in cortisol response (Bilbo et al., 2003). However no extensive research on glucocorticoid variation and association with behavioural phenotype in *P. campbelli* has been conducted. Thus, understanding the stress response in *Phodopus campbelli*, on both a

physiological and a behavioural level, will test and extend our current understanding of the generality of current inferences.

2.5 Behavioural Methods

Classic behavioural approaches to the study of stress use various arenas and environments to assess the motivation of a given animal to avoid a particular aversive consequence. However, there is no absolute standard in the design of these apparatus. The present review will focus on some of the most popular tests that were applied to the dwarf hamster in the present thesis including the open field, the holeboard, the elevated plus-maze, the light/dark box, and the territorial-intruder test (Table 1). These unconditioned models (Ohl, 2003), were chosen because the aim was to elicit a spontaneous behavioral result reflective of genetic background. Most of the following behavioural tests were originally developed for rats and were later adopted for mice. However, it should be noted that mice are not simply just ‘smaller rats’ but rather a different species that occupies a different ecological niche, resulting in different social behaviours, impulsiveness, and stress-coping strategies (Whishaw et al., 2001; Sousa et al., 2006). Thus the same apparatus can give different results between species as well as between sexes (File et al., 2000; Palanza, 2001; Rushen, 2000).

Table 1

Widely used tests to evaluate anxiety-related behavior in laboratory rodents

Name (abbreviation)	Origin/Investigator/ Species	Measurement	Principal indicators related to anxiety (simplified)
Open-Field (OF)	1934/Hall/rat	Emotionality (elimination)	+ defecation = + anxiety + locomotion = - anxiety
Holeboard (HB)	1962/Boissier and Simon/mouse	Exploration	+ locomotion = - anxiety + head-dips = - anxiety
Elevated Plus-Maze (EPM)	1955/Montgomery*/ rat	Fearfulness (height)	+ open arms = - anxiety + closed arms = + anxiety
Light/Dark box (L/D)	1981/Crawley/mouse	Fearfulness (illuminated area)	+ time in light = - anxiety + time in dark = + anxiety + transitions = - anxiety
Handling test	1913/Yerkes/rat	Resistant to human handling	+ aggression = + anxiety
Intruder Test (IT)	?	Territoriality (aggression)	+ aggression = + anxiety

* originally Y-maze

2.5.1 Open Field

The first and the most widely used test across vertebrate species is the open field. It was first described by Hall in (1936) (see Archer, 1973; Forkman et al., 2007 for a review). The open field can be generally described as a well illuminated area, several times larger than the home cage with subdivisions marked on the floor. The test relies on the observation that rats have a higher level of defecation and urination in a novel environment that does not provide immediate

protection from potential danger resulting in anxiety and emotional stress. More defecation and more time passed in the outer ring of the arena near the wall is interpreted as more anxiety or stress (Archer, 1973; Ohl, 2003). Hall proposed the original open field which was a circular, brightly lit area surrounded by a wall. Since then different types of open fields have been used, varying in terms of shape from circular to square, in terms of illumination from dimly lit to brightly lit, in terms of enrichment by offering objects or food, and in size. The testing procedure also differs widely: testing duration ranges from 2 minutes to several hours (Archer, 1973; Golani et al., 1999). Interestingly, Hall was aware of the limitation of group mean scores in emotionality. He wrote: '*It is quite possible that group averages might not hold up for individual cases, and that a rat which never voided during the period of observation was actually more upset than the rat which did defecate on one or more occasions*' (1934). As a result Hall (1938) declared that in order to validate emotionality measures, their relationship must be demonstrated using individual scores. Although evidence exists that the open field may be useful in detecting genetic or pharmacological effects on anxiety (Treit and Fundytus, 1988), some studies also report a lack of sensitivity for anxiety-modulations of this test (Saudou et al., 1994).

2.5.2 Holeboard

The holeboard test is basically an open field with holes on the floor. It was introduced by Boissier and Simon (1962). The test is based on the same premise as the open field, which is that anxiety is caused by unprotected and novel open space. The difference is that Boissier and Simon claimed that head-dipping provided a measure of exploration that was distinct form locomotor activity. However it was recently suggested that head-dipping and locomotion appear to be correlated (Klithermes and Crabbe, 2006a; 2006b). In studies using a battery of anxiety-related behavioral tests, the open-field and the holeboard are not usually used together. This apparatus was originally designed for mice. In is original form, the apparatus had 16 equally spaced 3 cm

diameter holes on a 40 cm^2 floor. The holeboard was modified later with only four holes in order to improve to discrimination between exploration and motor activity (Files and Wardill, 1975), because in its original version it was almost impossible for the mice to move around without touching a hole. The correlation in locomotor activity between the two versions was high but no correlation between versions could be made when "exploration" was measured (File, 2001). To be more precise, head-dipping seems to be a measure of escaping behavior rather than an exploratory behavior reflecting neophilia (Brown and Numes, 2008). Other versions were also developed. The modified holeboard consists of an open field separated in half by a vertical wall with holes. The animal is put in one side while a stimulus (e.g. food, another animal) can be put in the other (Ohl, 2003).

2.5.3 Elevated Plus Maze

One of the most frequently used tests for unconditioned anxiety in laboratory rodents is the elevated plus-maze (Sousa et al., 2006). This apparatus was first introduced, by Pellow and collaborators, in 1985 but its origin goes back to 1955 when Kay Cameron Montgomery proposed it as an elevated Y-maze. The test consists of an elevated, plus-shaped runway with two opposing arms being closed by walls and the other two arms being open. This test is based on the observation that rodents tend to avoid elevated areas (Montgomery, 1955) so avoidance of the open arms is interpreted as anxiety (Ohl, 2003). Again, different versions exist, for example the wall can be transparent or not, the area can be brightly lit or not (Sousa et al., 2006), but those factors seem to have an important influence on the outcome of the test (Pereira et al., 2005). Originally designed for rats, it is used extensively for mice as well (Belzung and Griebel, 2001; File, 2001). Moreover, it has been shown that the elevated plus-maze also allows controlling for locomotor activity (Rodgers et al., 1992), vertical activity, exploration, and provides indices of risk assessment and decision making (Rodgers and Dalvi, 1997).

2.5.4 Light/Dark Box

Again making use of the conflict between a rodent's motivation to explore a novel environment and its avoidance of brightly lit areas, the light/dark box (also referred to black/white box or light/dark exploration) comprises one aversive (light) and one less aversive (dark) compartment. In its original setup, the dark compartment was smaller than the lit one and both compartments are separated by a partition containing an opening (Crawley and Goodwin, 1980). There are variations in the size of the apparatus and its sectors that are used throughout the literature (Bourin and Hascoët, 2003). An increase in transitions from the light to the dark area, without an increase in spontaneous locomotion, is considered to reflect anxiolytic activity. It is interesting to note that this effect is only observed in certain strains of mice or with certain drugs (Bourin and Hascoët, 2003).

2.5.5 Intruder Aggression Test

Aggression in the rodent model has a strong emotional component and is influenced by fearfulness and therefore could be sensitive to the HPA axis and stress variations (Blanchard and Blanchard, 1989; Wommack and Delville, 2007). The organization of the intraspecific agonistic behavior of the rats (*Rattus norvegicus*), mice (*Mus musculus domesticus*) and the Golden hamster (*Mesocricetus auratus*) is well documented (Koolhass et al., 1980; Blanchard et al., 2003). The comparisons of these three species of rodents show similarity in their aggressive behaviors but also differences (Blanchard et al., 2003). For example, hamsters compared to rats and mice are particularly prone to becoming non-aggressive and submissive following defeat (Huhman, 2003). The territorial intruder-test is a classic test to evaluate intraspecific agonistic behavior and was conducted previously on dwarf hamsters (Wynne-Edwards and Lisk, 1987; Hume and Wynne-Edwards, 2005). Typically, a conspecific of the same sex is introduced into the home cage of the focal host. During the time of the trial, dyadic aggressive behaviors are

recorded. The trial ends when the time chosen by the researcher prior to the experiment is up or if at least one member of the dyad is about to get injured by the other. Different versions of this test also exist depending of the objective of the experiment. For example, an investigator could remove the pups in studying maternal aggression. In the specific case of dwarf hamsters (*Phodopus* spp.), the following measures are usually recorded; the presence or absence of attack with bites, the number of boxing matches, and the number of times a hamster fled or assumed a submissive 'on-back' position (Hume and Wynne-Edwards, 2005).

2.6 Adaptive value of individual variation in the stress response

The analysis of individual hormonal variation in different areas of biology has been gradually increasing over the past few years (Williams, 2008). In the emerging field of evolutionary endocrinology, data suggesting that hormones are critically involved in adaptation and evolution of complex traits is beginning to be recognized (Zera and Harshman, 2001; Sih et al., 2004). Individual variation in endocrine regulation affects response to environmental change and thus affects an animal's fitness (Dufty et al., 2002; Zera et al., 2007) through inter-individual variation in stress coping styles (Korte et al., 2005; Koolhaas et al., 2007). In addition, an individual's phenotype can be modified during its lifetime (Piersma and Drent, 2003). Thus, the greatest challenge in measuring stress is understanding inter-individual variation in the stress response (Moberg, 2000; Williams, 2008). A multidimensional approach to the study of the individual stress response, including physiological and behavioural measurement, is needed.

2.7 Hypotheses

To investigate the relationship between plasma glucocorticoid concentration and emotion-related behaviour in *Phodopus campbelli* on an individual basis, this thesis presents the result of two major studies. The two experiments were designed to test the following two hypotheses:

1. **The plasma cortisol response to acute physical restraint stress in *P. campbelli* is homogenous across individuals.**

2. **The individual plasma cortisol response to acute physical restraint stress in *P. campbelli* is reliably predicted by individual behavioral responses to emotion-related stress tests.**

The remainder of the thesis is structured as two results chapters (Chapters 3 and 4) corresponding to tests of these two hypotheses, followed by a general discussion (Chapter 5).

Chapter 3

Individual variation in cortisol responses to acute 'on-back' restraint in an outbred hamster

3.1 ABSTRACT

An outbred species of dwarf hamster (*Phodopus campbelli*) was used to assess between-individual variability in the response to, and recovery from, a one-time stressor of 6 min of physical restraint in a subordinate, on-back, position. Four repeated plasma samples were drawn under home-cage isoflurane anesthesia from 33 males and 38 females 50 min before, and then 10, 60, and 120 min after the stress onset. Plasma cortisol concentrations were higher in females than males but there was no evidence for a sex difference in response to the stressor. The expected cross-sectional increase (~50 ng/ml) in response to the stressor, followed by recovery, was seen. However, there was extensive individual variation, ranging from no reaction to continuous decline from the initial to the final sample. Results were expressed in four ways (absolute concentration, relative concentration, and area under the curve relative to ground and relative to the stress-induced increase) and also standardized and subjected to hierarchical cluster analysis. Clusters failed to effectively partition the between-individual variation and did not cluster by sex, age, or housing conditions. The current study cautions against ignoring individual differences, and suggests that outbred animal models might be particularly relevant to understanding stress-related pathological conditions.

3.2 INTRODUCTION

One of the greatest challenges in understanding the complexities of the stress response is the difficulty encountered when trying to understand the extent of inter-individual variation (Moberg, 2000; Young et al., 2004). Glucocorticoids are responsive to stress and the majority of studies use the concentration of cortisol or corticosterone as a proxy for the magnitude of stress experienced by the individual (Erikson et al., 2003; Korte, 2001). However, the range of glucocorticoid concentrations in a cross-sectional analysis of any population can be large, and researchers have found advantages in experimental designs that use repeated measurements of glucocorticoid concentrations (Amario et al., 2004; Jones et al., 2000; Ramsay and Lewis, 2003). Such studies suggest that there is heterogeneity in the duration of time required for corticosteroid concentrations of mice (Veenema et al., 2003), rats (García and Amario, 2001; Meaney et al., 1993; Mormède et al., 2002) and humans (Mormède et al., 2002; Ramsay and Lewis, 2003; Richter et al., 1996) to return to their resting levels. Understanding the basis for the variation in corticosteroid dynamics may be important in explaining individual susceptibilities of the immune system (Barriga et al., 2002; Bilbo et al., 2003; Carlton et al., 2001; De Kloet et al., 1998; Korte, 2001), growth and development (Brown and Nestor, 1974; Castanon et al., 1994; Tsigos and Chrousos, 2002), and emotional states (Amario et al., 2004; Erikson et al., 2003; De Kloet, 2004; Roy et al., 2001; Young et al., 2004).

The current study was designed to quantify and partition individual cortisol responses to a standardized stressor in an outbred population of rodents held under laboratory conditions. The model system chosen was the Djungarian dwarf hamster, *Phodopus campbelli*, because this population retains behavioral characteristics of the wild ancestral population from which it is derived, including entrainment of activity to the light-dark cycle (Wynne-Edwards et al., 1999; Wynne-Edwards, 1998), and has complex social behavior that includes extensive paternal care

(Wynne-Edwards, 1998; 2003; Wynne-Edwards and Lisk, 1989). During aggressive encounters, a submissive animal is restrained in an on-back position by a dominant animal (Hume and Wynne-Edwards, 2005; Wynne-Edwards and Lisk, 1987; 1989), so 'on-back' restraint was chosen as an ecologically valid stressor. In addition, a method of home cage anesthesia has been validated for these hamsters to allow the collection of repeated blood samples without handling- or anesthesia-induced changes in cortisol concentration (Reburn and Wynne-Edwards, 2000). Finally, the dominant glucocorticoid in these hamsters is cortisol (Reburn and Wynne-Edwards, 2000), like humans but unlike rats and mice. Thus, both males and females were subjected to a one-time on-back restraint and had plasma sampled four times, from before the stressor to after the recovery. The intention was to apply a range of current analytical approaches to determine which methods were most effective for comparison across individuals.

3.3 METHODS

3.3.1 Animals

Dwarf hamsters of the species *Phodopus campbelli* were outbred descendents of wild-caught individuals sampled from a breeding colony maintained at Queen's University (Wynne-Edwards, 2003). All animals were cared for in accordance with the Canadian Council on Animal Care (Olfert et al., 1993) under Queen's University protocol 041. Litters were weaned at 18 days into same sexed sibling groups of one to three animals. Hamsters were housed in polycarbonate cages (27 cm long x 21 cm wide x 14 cm high; Nalgene caging, Nalge-Nunc, Rochester, NY) with food (Lab Diet 5001 rodent diet; PMI Nutrition International, Brentwood, MO) and tap water available *ad libitum*. Bedding was 2 cm of wood shavings (Emerald Peak, Kingston, Ontario). Ambient temperature was 18 $\pm$ 1°C to reflect the burrow temperature of the wild environment (Wynne-Edwards, 2003). Photoperiod was 14L:10D with lights off at 19:00h. Males (n = 33) ranged from

34 to 84 days old (mean ± SEM =58.3 ± 2.3 d; weight range 20.6 - 41.5g; mean weight = 32.7 ± 0.8g) and females (n = 38) ranged in age from 34 to 106 days old (65.2 ± 2.6 d; 20.6 - 36.3 g; 27.6 ± 0.6g). Only one hamster from any like-sexed sibling group was used for this study. In practice, only one male and one female had been previously housed in a group of three (which can suppress estrous cyclicity; Erb et al., 1993) and all other hamsters were previously housed in sibling groups of two or alone. As vaginal smears do not predict behavior, estrous cycles in this species can only be tracked by exposure to a male to assess sexual behavior (Wynne-Edwards et al., 1987). Thus, female cycle stage was not monitored to avoid the additional stress of introduction to an unknown male. To prevent endocrine changes associated with familiarity with the restraint stressor, all individuals were tested only once.

3.3.2 Cage modifications

To minimize stress, animals were transferred to another room under identical environmental conditions at least 24 h before sampling (Tuli et al., 1995). One day of isolation in an individual cage removes cortisol differences due to social housing under normal as well as stressful conditions (Reburn and Wynne-Edwards, 2000). Home cages that allow small mammals to move freely while they are repeatedly sampled were used for blood sampling (Reburn and Wynne-Edwards, 2000). The cages were modified in three ways. 1) The steel lid and food hopper was replaced with a 3 mm Plexiglas™ lid with five exhaust ports and openings for a 250 ml water bottle with a stainless steel sipper tube (Nalgene) and a glass 'L shaped' rod. 2) Bedding was reduced to 1 cm to minimize absorption and retention of anaesthetic, and food was provided on the floor of the cage. 3) Air was continuously delivered by individual air pump (Elite 803, Hagen Inc., Montreal, Quebec) through a 6 mm diameter tubing system of clear PVC™ (Tygon, S50 HL, Norton Performance Plastics Corp., Akron, OH) ending with the glass 'L shaped' rod (Pyrex® glass tubing) which cleared the bedding by 2 cm (Reburn and Wynne-Edwards, 2000) and

provided a minimum air flow of 2.9 l/min (Brooks et al., 2005; Schum and Wynne-Edwards, 2005). Nalgene 'quick disconnects' allowed the operator to remotely switch from air to anaesthetic behind an opaque curtain.

3.3.3 Anesthesia

The anaesthetic was 3.5% Isoflurane® (1-chloro-2,2,2-trifluoroethyl difluoro methyl ether) vaporized (Fluotec, Fraser Sweatman Inc., Buffalo, NY) in medical grade oxygen (O_2) at a flow rate of 3.5 l/min. Isoflurane is a self-administered, inhaled, ether based-anaesthetic that has low toxicity and properties of quick induction and recovery (Flecknell, 1987). Furthermore, this anaesthetic is a short-term amnesiac (Ono and Maeshima, 1998). Isoflurane induces a surgical plane of anaesthesia in these hamsters within 60 seconds of exposure and recovery usually occurs within 40 seconds (Reburn and Wynne-Edwards, 2000). The room contained 4 to 8 cages at any time. All animals were anaesthetized simultaneously and held under anaesthesia until all blood samples had been collected (6 to 12 min exposure).

3.3.4 Plasma collection

Under this brief isoflurane anesthesia, animals had a small plasma sample taken from the lateral retro-orbital sinus (Timm, 1989). Retro-orbital sinus sampling is the preferred technique for collecting blood in this small species (< 40 g) with short limbs (1.2 cm), a short tail (7 to 9 mm), and thick pelage that makes peripheral vasculature inaccessible. Each sample consisted of 150µl of whole blood (2 x 75µl heparinized microcapillary tube, Fisher Scientific). In total, four samples were taken from each individual (t_1 to t_4). Samples larger than this (up to 1200µl as a single sample, or up to 12 samples over 24h) are routine - hematocrit is well defended and both social and sexual behavior remain normal (Edwards, et al., 1995; McMillan and Wynne-Edwards 1998, 1999; Reburn and Wynne-Edwards 1996, 2000; Wynne-Edwards et al., 1987). The first

sample was collected at 1330h and the last at 1630h so that all samples would be during the inactive, light-phase of the photoperiod. These hamsters spontaneously arouse and begin nocturnal activity approximately 2h after the last sample (30 min before lights-off at19:00h; Wynne-Edwards et al., 1999), with the expected awakening surge of cortisol at that time (Reburn and Wynne-Edwards, 1999). Microcapillary tubes were centrifuged for 2 minutes in an IEC Model MB microhematocrit centrifuge at 13,460 x g. Hematocrit was determined for each microcapillary, and no animals were excluded from further sampling on the basis of a drop in hematocrit. The plasma fraction was transferred into a 0.25ml microcentrifuge tube, capped and stored at -20°C until assayed for hormone content.

3.3.5 Restraint stress

Cortisol response was measured by exposing each hamster to a forced on-back restraint for 6 min. Each restraint was individually adjusted to preclude movement. The on-back position simulated a subordinate social posture, which typically results in immediate urination (Hume and Wynne-Edwards, 2005; Reburn and Wynne-Edwards, 2000; Wynne-Edwards and Lisk, 1987). No attempt was made to assess the social dominance of individuals before testing since this study focused on population variability. Fifty-two minutes after the first plasma sample, each hamster was moved into a clean transition cage for two minutes. Each animal was then placed in a clear polyfilm rodent restraint cone (Harvard Apparatus Inc, Holliston, MA), immobilized, inverted, and attached to a Plexiglas™ platform with a 50 mm binder clip. At least three, and up to six hamsters were put in restraint with a 30 second interval between each. After 6 min in the cone, animals were put back into their modified cages in the same order. All hamsters were then anaesthetized at the same time to take the post-stress plasma sample.

3.3.6 Radioimmunoassay

Cortisol was measured using a ^{125}I Cortisol double antibody kit (KCOD1-2) from Diagnostic Products Corp. (Intermedico, Markham, Ontario). Recommended procedures were followed exactly except that the volumes of samples, standards, and reagents were reduced by half (Reburn and Wynne-Edwards, 1999; 2000). Plasma samples were assayed in duplicate at 10µl. The range of sensitivity of the assay was calculated from six assay runs resulting in 33.2 pg/tube at 95% and 7.1 ng/tube at 5% binding for a measured range of 3.3 ng/ml to 0.7 µg/ml. All plasma samples fell within the sensitivity range of the assay (22-368 ng/ml = 65-18% binding). Three human-serum based immunoassay controls (Intermedico, Markham, Ontario) were used to check the reference range of the assay, and two *Phodopus campbelli* blood pools served as internal controls. Intra-assay and inter-assay coefficients of variation for the pooled samples were 6.1% and 15.6% respectively. To eliminate inter-assay variability as a source of within individual measurement error, all samples for any individual were always quantified within a single assay run.

3.3.7 Statistical analyses

All statistical analyses, except the hierarchical cluster analyses, were performed with JMP version 5.0.1 software (SAS Institute, Cary, NC). Significance was taken at $p < 0.01$ to recognize the multiple comparisons that were planned. Cortisol data were log-transformed to achieve homogeneity of variance (Levene's *F*-test). Multivariate ANOVAs with repeated measures and Tukey-Kramer HSD as the *post-hoc* test were used to analyse absolute and relative cortisol concentration across time points. Sex difference in the initial samples was compared using a Students *t*-test. Linear relationships between putative baselines (t_1 and t_4) as well as between t_1 and age were calculated by Pearson correlation coefficient (*r*). Additionally, two areas under the curve (*AUC*) were calculated to assess changes over time (Pruessner et al., 2003). First, the area under the curve with the respect to ground (AUC_G), reflected the absolute change over time and

was calculated as the sum of each trapezoid area across the four time points. Second, the area under the curve with respect to the increase (AUC_I) reflected the relative intensity of change over time and was calculated as the sum of each trapezoid area between time points minus the rectangular area formed by the time points on the *x* axis and the value at the lowest point on the *y* axis (cortisol concentration).

To identify common patterns across individuals based on the shape of their response curves, individual cortisol response was compared by hierarchical cluster analysis using an average linkage between-group method and the Pearson product-moment correlation r_{jk} coefficient (where *j* is the individual value and *k* the time point; SPSS version 10.0, Chicago, IL). This method minimizes the variance within clusters and maximizes the difference between them. Data were standardized using Z scores to minimize size displacement in the data profile and hence reflect similarity in cortisol curves rather than concentrations. The analysis used the distance between samples (time) as attributes to reflect similarity in cortisol curves (Romeburg, 1984; Sokal and Michener, 1958). The clusters were formed at the maximum width of range between coefficients (Kaufman and Rousseeuw, 1990; Romeburg, 1984). The within-group coefficient required to determine statistical significance was conservative and fixed at 0.60 to identify a relationship of moderate intensity (Scherrer, 1984).

3.4 RESULTS

3.4.1 Cortisol concentration in the first sample (t_1)

Regressions between age and baseline (t_1) were not significant in either sex (males: $t = 0.19$, $r = 0.03$, $p = 0.85$; females: $t = -1.49$, $r = 0.24$, $p = 0.14$), suggesting that age at the time of sampling did not affect the variation in the t_1 sample (Fig. 2). Likewise, body mass did not predict cortisol concentration at t_1 (males: $t = 0.35$, $r = 0.06$, $p = 0.72$; females: $t = -1.08$, $r = 0.17$, $p = 0.29$).

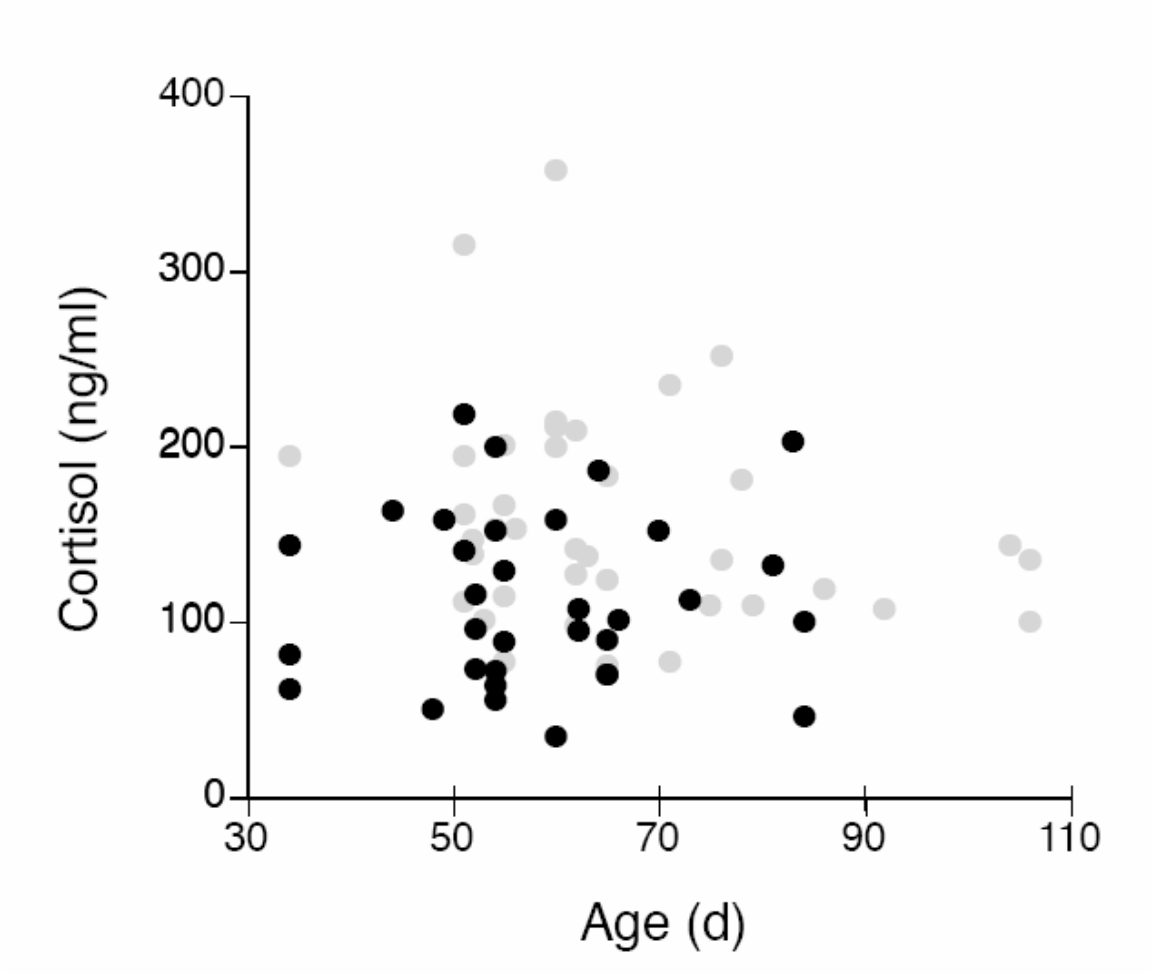

Figure 2. Individual plasma cortisol concentrations 50 min before stress onset (t_1) plotted against age for females (gray circles) and males (black circles). Linear regressions were not significant and are provided in the results.

Finally, group housing versus solitary housing from weaning until before the 24h of isolation and standardized testing) did not affect the cortisol concentration at t_1 (males: t (31) = 1.88, p = 0.07; females: t (36) = 1.04, p = 0.31) and recovery (t_4-t_2) (males: t (31) = 1.24, p = 0.23; females: t (36) = 1.54, p = 0.13).

3.4.2 Cortisol response to the standardized stressor

As expected, main effects of acute stress across repeated samples (F (3,68) = 38.77, $p < 0.001$) and between sexes (F (1,69) = 15.27, $p < 0.001$) were seen (Fig. 3A). For the majority of animals (55/71), the highest concentration was in the sample immediately following the stressor (t_2). The pattern was consistent in both sexes (males: F (3,30) = 21.72; females: F (3,35) = 17.36, all $p < 0.001$) with cortisol concentration significantly higher at the second time point (t_2) than in the three other sampling times (t_1, t_3 and t_4), although cortisol concentration was significantly higher in females than males at each time point (all $p < 0.001$). The average reactivity (t_2-t_1) of 50 ng/ml (males 50.8 ± 6.8 ng/ml, females 48.3 ± 8.4 ng/ml; t (69) = 0.23, p = 0.82) represented a 45% increase in males and a 30% increase in females. As well, cortisol amplitude (t_2 – (average of t_1, t_3, t_4)) for each individual) did not differ for the two sexes (males 54.8 ± 7.0 ng/ml, females 49.2 ± 6.7 ng/ml; t (69) = 0.58, p = 0.57). However, the range of absolute concentrations at each time point was large (SD range = 49 - 68 ng/ml) and the coefficient of variation was as large as 63% in some samples (Table 2). For all time points, variances were statistically homogenous in both sexes.

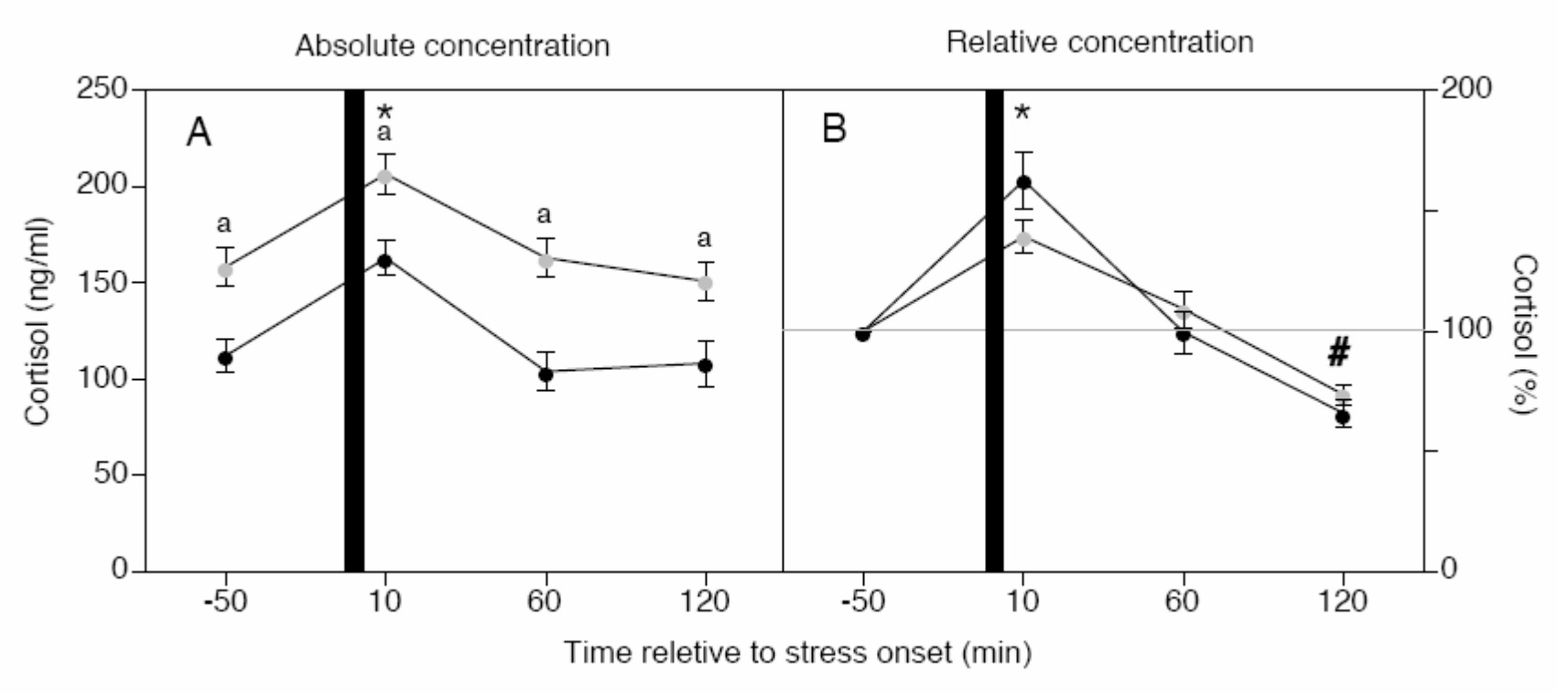

Figure 3. Effect of a 6 min on-back restraint on plasma cortisol. A) Absolute cortisol concentration (ng/ml) and B) Relative cortisol concentration (% of t_1 concentration) for females (gray circles: $n = 38$) and males (black circles; $n = 33$). Samples were taken 50 min before stress onset (t_1), 10 min after stress onset (t_2), after 60 min of recovery (t_3), and after 120 min of recovery (t_4). The shaded bar indicates the restraint. Results are expressed as mean ± SEM. * higher than t_1; # lower than t_1; [a] difference between sexes; all $p < 0.001$.

Table 2

Plasma cortisol concentrations (ng/ml)

sampling time	sex	range	n	min	max	mean	SEM	SD	CV %
stress – 50 min (t_1)	*all*	342	71	34	358	136.9	7.2	60.7	44.4
	males	184	33	34	218	112.1	8.5	49.1	43.8
	females	283	38	75	358	158.4	10.1	62.3	39.3
stress + 10 min (t_2)	*all*	290	71	78	368	186.3	7.5	60.1	32.2
	males	178	33	78	257	162.9	9.3	53.1	32.6
	females	280	38	88	368	206.6	10.5	64.6	31.3
stress + 60 min (t_3)	*all*	258	71	33	291	135.6	8.0	67.0	49.4
	males	201	33	33	233	104.0	9.4	57.1	54.9
	females	231	38	61	291	163.0	10.3	63.4	38.9
stress + 120 min (t_4)	*all*	318	71	22	340	131.0	8.1	68.1	52.0
	males	318	33	22	340	108.2	11.9	68.2	63.0
	females	248	38	27	275	150.9	10.1	62.4	41.4

3.4.3 Within individual response to the standardized stressor

To assess whether the pattern was the same in all individuals in spite of the different absolute concentrations, cortisol concentrations were also expressed as a proportion of the cortisol concentration in the sample 1 h before the stress (t_1). Following this calculation, there was still a significant cortisol increase from t_1 to t_2, but also a significant decrease two hours after stress (t_4; F (3,68) = 36.2, $p < 0.001$; Fig. 3B). Again, this pattern was present in both sexes (males: F (3,30) = 17.2; females: F (3,35) = 20.0, all $p < 0.001$). However, in this case, males and females did not differ ($t = 1.74$, $p = 0.89$; $t = 0.85$, $p = 0.40$; $t = 1.12$, $p = 0.27$, respectively for t_2, t_3, and t_4). There was a linear relationship between the absolute concentration for the pre-test baseline level (t_1) and the concentration after the putative return to that baseline (t_4) across all individuals ($r = 0.59$, $p < 0.001$) as well as for both sexes (males: $r = 0.50$, $p = 0.003$; females: $r = 0.55$, $p < 0.001$; Fig. 4).

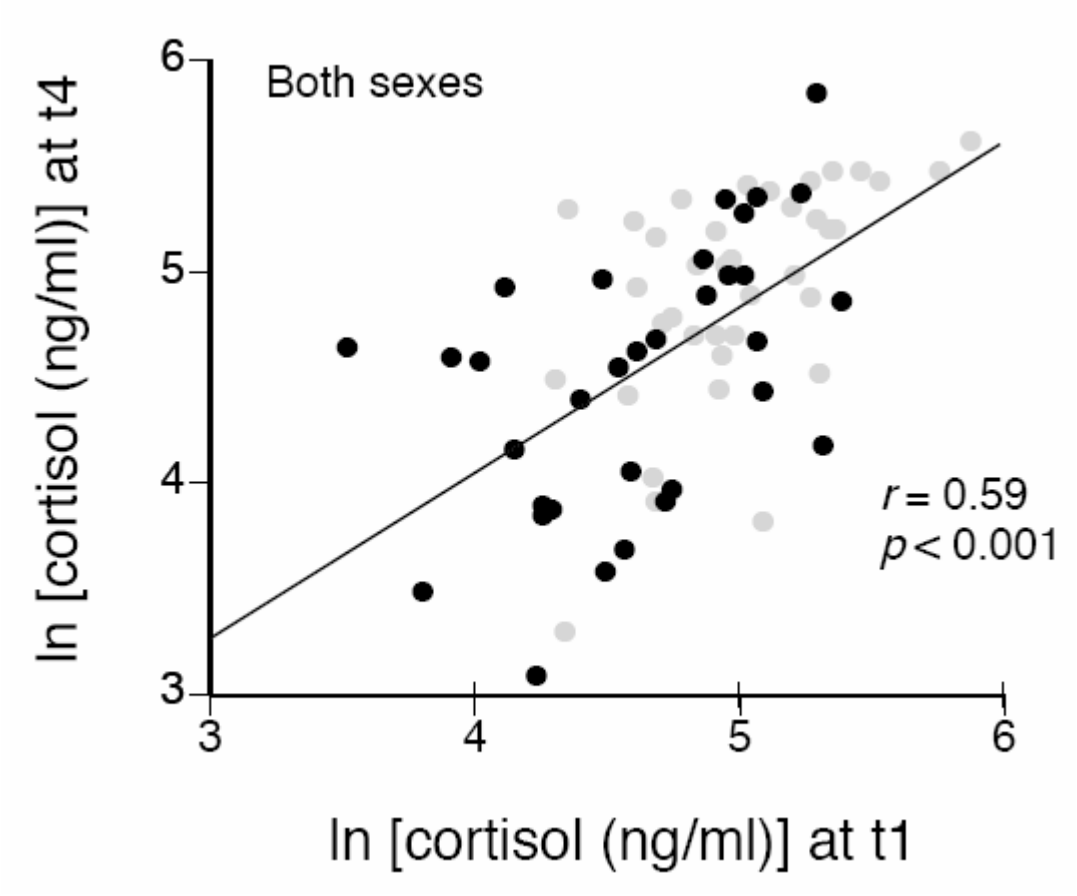

Figure 4. Log-transformed plasma cortisol concentration 120 min after stress onset (t_4) plotted against log-transformed plasma cortisol concentration 50 min before stress onset (t_1) for females (gray circles) and males (black circles). The linear relationship across both sexes is shown. Regression within each sex is provided in the text.

3.4.4 Area under the curve comparisons

Finally, the area under the curve (*AUC*) was calculated both for absolute hormone concentrations (AUC_G) and for relative hormone concentrations (AUC_I) (Table 3). These measures were intended to incorporate all four cortisol determinations for an individual into a single variable integrating the amplitude of the response as well as the time to recovery. Both measurements were controlled for co-variation with age and body mass. As expected the AUC_G, but not the AUC_I, was significantly higher in females than males ($t = 3.99$, $p < 0.001$). However, the coefficient of

variation for AUC_G was not reduced relative to individual samples in Table 2 and the coefficient of variation for the AUC_I was over 200%. In fact, there were 23 individuals (32.4%) with a negative AUC_I. Thus, although the cross-sectional sample showed the expected pattern of cortisol increase following the stressor and recovery over the next two hours, there was considerable inter-individual variability.

Table 3

Area under the curve (ng/ml)

AUC	sex	range	n	min	max	mean	SEM	SD	CV %
[1]AUC_G	*all*	804	71	172	976	455.8	20.3	170.9	37.5
	males	569	33	172	741	377.0	24.4	140.0	37.1
	females	756	38	219	976	524.3	27.1	167.2	31.9
[2]AUC_I	*all*	478	71	-168	310	45.2	12.7	106.9	236.5
	males	368	33	-168	198	40.6	15.7	90.1	221.9
	females	462	38	-152	310	49.2	19.6	120.6	245.3

[1] AUC_G: area under the curve with respect to the ground corresponding to the sum of each trapezoid area between
the 4 time points

[2] AUC_I: area under the curve with respect to increase corresponding to the sum of each trapezoid area between
time points minus the area under the value of the lowest time point.

3.4.5 Cluster analysis

In an effort to further investigate this variability, a hierarchical cluster analysis was used to search for common patterns through time across individuals. In general, the cluster approach suggested that discrete patterns across individuals could not be accurately assigned with our sample of 71 individuals. For example, the large range of absolute cortisol concentrations resulted in a six cluster model (Fig. 5) with a within group coefficient of correlation of only 0.37 after 66 iterations. Reaching a threshold coefficient greater than 0.60 required ten clusters in that particular model (r_{jk}= 0.62). For relative cortisol concentration, the maximum distance between coefficients arranged the output in a five cluster model with weak explanatory power (66

iterations; $r_{jk} = 0.33$). In these five groups, the peak could occur at t_2 or t_3, the nadir could occur at t_1, t_3 or t_4, and recovery following stress was not always monotonic (Fig. 6).

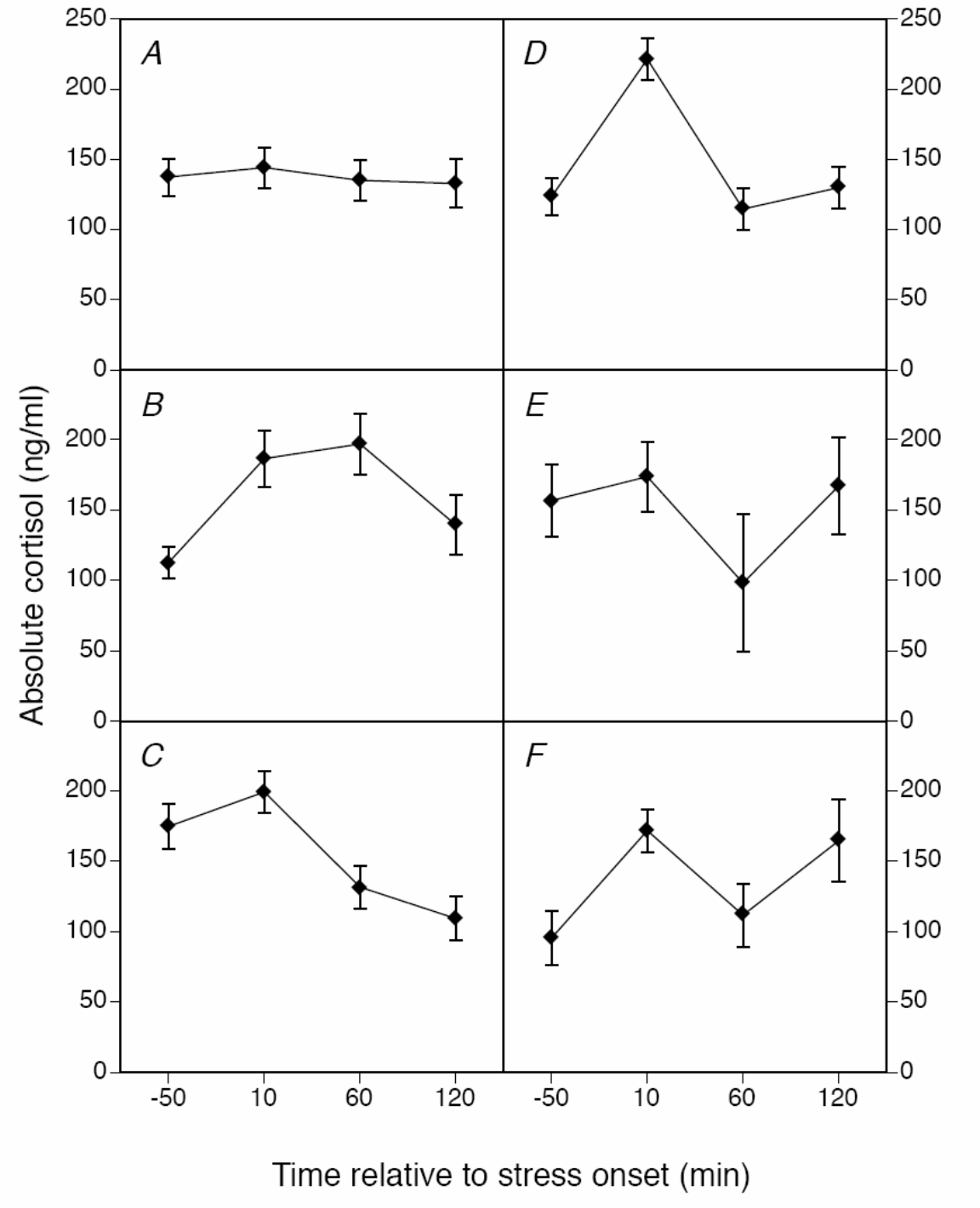

Figure 5. Cortisol concentration (ng/ml ± SEM) for each of six clusters (A-F), (n_A = 15; n_B = 11; n_C = 20; n_D = 15; n_E = 2; n_F = 10). The *x*-axis represents sampling time and the *y*-axis plasma cortisol concentration (ng/ml). Samples were taken 50 min before (t_1), 10 min after (t_2), 60 min after (t_3) and 120 min after (t_4) a 6 min stress onset.

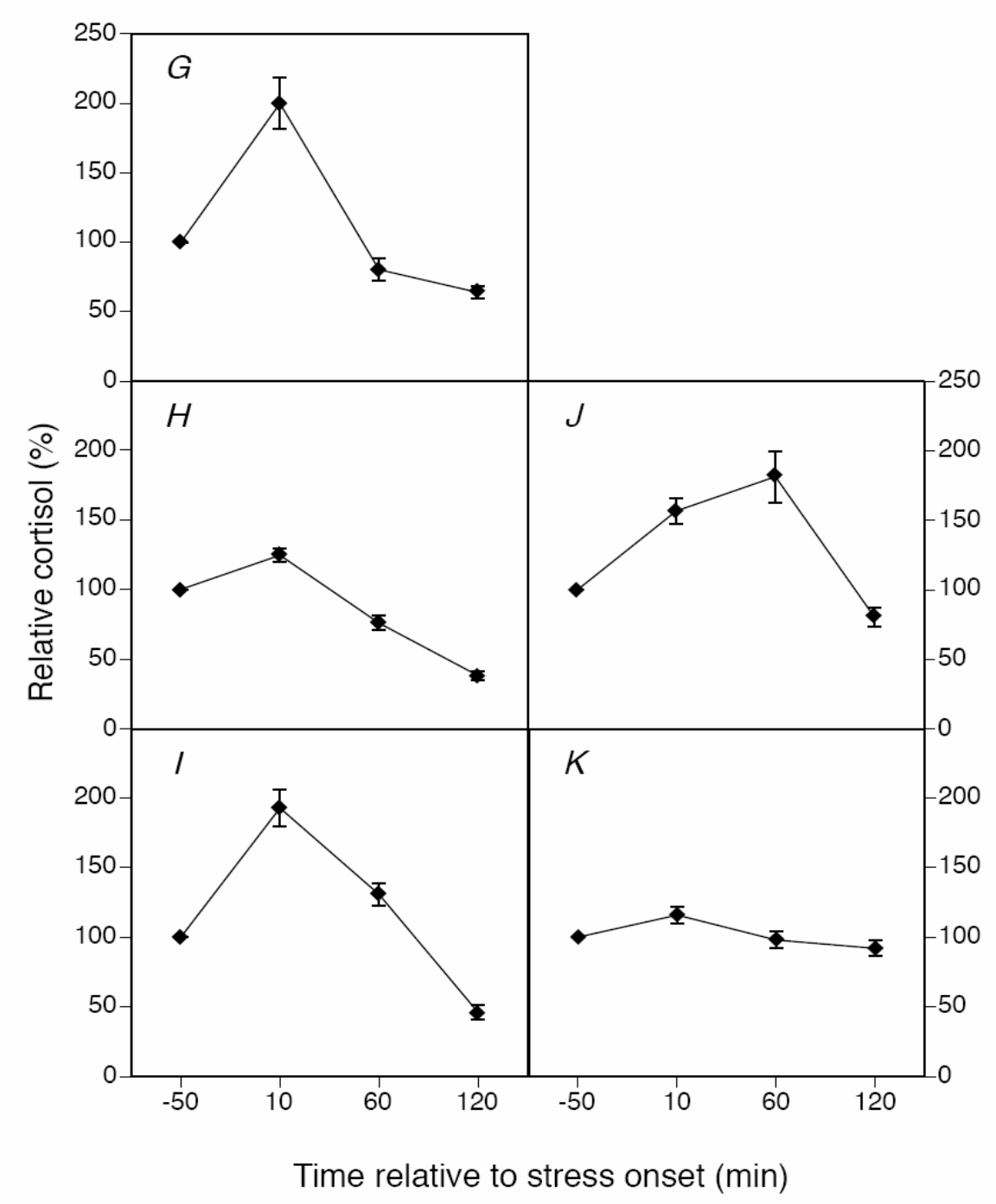

Figure 6. Relative cortisol concentration (% of t_1 ± SEM) for each of five clusters (G-K). (n_G = 17; n_H = 13; n_I = 7; n_J = 10; n_K = 24). The x-axis represents sampling time and the y-axis percentage. Samples were taken 50 min before (t_1), 10 min after (t_2), 60 min after (t_3) and 120 min after (t_4) a 6 min stress onset.

A threshold coefficient of 0.60 required ten, nine, and eight clusters in models considering concentrations relative to t_1, t_2 and t_4. The cluster analysis also failed to find any profile specific to either sex, or to animals housed alone *vs* with a like-sexed littermate sibling, or any profile that separated adolescent from adult hamsters. For example, the youngest animals (<50 d) were distributed among 5 different clusters.

3.5 DISCUSSION

Although the population showed the expected increase in cortisol in response to the on-back restraint, the primary conclusion from this study of 71 adult hamsters was that individual variability was exceptionally large. Remarkably few individuals showed the average pattern of a 50 ng/ml cortisol increase in response to the stressor followed by a recovery to baseline. Attempts to improve homogeneity of the response by expressing hormone concentrations relative to initial values, or summarizing the overall response through area under the curve measures, were not successful.

When results were expressed relative to the initial sample (t_1) the concentration at t_4 was significantly lower than that putative baseline. Such calculations relative to baseline are more sensitive to heterogeneous groups, especially when there is a substantial individual variation in baseline level (Wolf et al., 2002). Thus, two hours after the end of a stressful event, cortisol concentration in these hamsters reached a "relief" phase (Michaud et al., 2003) that was not detected in the cross-sectional analysis of absolute concentrations. This recovery time was similar to recovery latencies for mice and rats (Beane et al., 2002; Cordero et al., 2003; Gärtner et al., 1980; Hess et al., 1969; McCormick et al., 2005; Veenema et al., 2003) although glucocorticoid

half-life can differ profoundly between species or even subspecies (Alexander et al, 1993; García et al., 2000; Windle et al., 1998a).

A surprisingly large 22.5 % of individuals reached their peak elsewhere than t_2. In cases where t_3 exceeded t_2, this might be explained by individual differences in the time required to reach maximum concentration. In laboratory rats, maximal glucocorticoid concentration is normally achieved approximately 20-30 min after stress onset (García et al., 2000) although important individual differences in HPA recovery after stress remain (García and Amario, 2001). In the current study, the interval between initial handling by the investigator, and the anesthetised sample after the stress, was 10 minutes. Alternate explanations for peak concentrations at t_1, or t_4 are not known. During 120 minutes of confinement, *P. sungorus* females had higher cortisol concentration at 120 minutes than at 10 minutes although male *P. sungorus* and both male and female *P. campbelli* had declined in spite of the persistence of the confinement stress (Bilbo et al., 2003). Although this study also took a sample 120 minutes after the onset of the stress, it differed from the Bilbo et al. study because the stress was acute (and contained the social component of on-back subordination) and because the samples at t_3 and t_4 measured recovery whereas the other study maintained the confinement.

Female rodents typically release more adrenocorticotropic hormone (ACTH) and glucocorticoids in response to stressors than males (Bilbo et al., 2003; Windle et al., 1998a; Young et al., 2004). Hence, even though stage of the estrous cycle was not controlled, female cortisol concentration was higher than male cortisol concentration with an equivalent variance. Both estrogen and progesterone affect the HPA axis and corticosteroid binding globulin (Lunga and Herbert, 2004; Seale et al., 2004; Young and Altemus, 2004). In addition, oxytocin differentially influences the HPA axis in males and females (De Kloet, 2004) and facilitates social interaction and pair bonding (Carter, 1998). Finally, there is a negative association between

testosterone and the HPA axis (Viau and Meaney, 1996). However, in spite of these potential sources of sex differences, the current study found no sex differences in cortisol amplitude, reactivity, clustering, or variance. Both sexes reacted similarly to the stressor and intra-sex variability was larger than inter-sex variability.

The post-stress impact of glucocorticoids on the individual organism was also assessed by *AUC* (García et al., 2000). This measure integrated the duration and the peak of the glucocorticoid response. If peak concentration after stress can move during a recovery period, such as when individuals show wide variation, the *AUC* is particularly useful (García et al., 2000). *AUC* measures also revealed substantial individual variation, especially for AUC_I, which emphasizes changes over time more than the AUC_G. This variation in AUC_I reflected the dispersion of positive and negative values in the population. Twenty-three animals had negative values for AUC_I indicating that their cortisol concentration was reduced relative to t_1 at other sample times. Age and body mass were co-variates in the analysis. Thus, expressing the results as area under the curve did not reduce individual variability.

Likewise, the cluster analyses suggested a continuum of variation across the population rather than a set of well-defined clusters for either absolute or relative cortisol concentration responses. There was no evidence that dichotomizing the hamsters by sex, or previous housing alone vs with a like-sexed sibling, or even focussing on the youngest hamsters (adolescents ≤ 50d) contributed to the formation of any cluster. Other aspects of individual behavior, such as dominance status within the like-sexed littermate pair, might have contributed to the variability seen among individuals. However, in the absence of a small number of clearly defined clusters it remains unlikely that such a behavioural distinction would have explained much of the variation.

Finally, there was a positive linear correlation between the concentrations for each individual at t_1 and t_4 (the two putative baseline samples) which suggested that a high cortisol

hamster before the stress challenge tended to remain high two hours after that acute stress whereas a hamster with a low cortisol concentration at t_1 had a tendency to stay low at t_4. This association, however, also still left much of the variability (65%) unexplained.

One important factor expected to contribute to these individual differences is position in a circhoral glucocorticoid cycle. These cycles affect 'baseline' and thus the response to stress can vary from a minimal response during a falling phase to a fourfold increase during a rising phase (Windle et al., 1998a; Young et al., 2004). Additional variation might be a result of multiple cortisol release events (Pignatelli et al., 1998; Windle et al., 1998b) and individual variability in the activation of these multiple release events (Richter et al., 1996). However, the experimental design did control the timing so that all samples were obtained during the inactive, light, phase of the 24 h cycle and the last sample was 2 h before the arousal increase in cortisol concentration (Wynne-Edwards et al., 1999; Reburn & Wynne-Edwards, 1999). Thus, the sample at t_1 represented a trough in the circadian cortisol rhythm that should have allowed ample opportunity for a response to the stressor.

Differential stress perception and integration among individuals is also likely to have contributed to individual variability. However, the stressor was a complete physical restraint, adjusted for each individual, that forced the hamster into a socially subordinate, on-back, position of defeat. Thus, all hamsters were expected to perceive the intervention as highly stressful. Corticosteroid levels depend on stressor intensity (García et al., 2000), and when the stressor is more intense the corticotrophin-releasing factor (CRF) mRNA increases in the paraventricular nucleus of the hypothalamus (PVN) for a longer period (Amario et al., 2004). Consequently, individual hamsters with a relatively high concentration of plasma cortisol 2 h after the stressor might be explained by persistence of ACTH stimulation due to bidirectional feedback between the PVN and the brainstem (Huether, 1996). Another source of species variation could come from

polymorphism in corticosteroid receptors, as the receptors certainly vary on a species level (Ronchi et al., 1998; Sutanto and De Kloet, 1994), or corticosteroid binding-globulin diversity (Breuner and Orchinik, 2002). However, the biological substrate responsible for individual differences in recovery of the HPA axis is unclear. For example, differential susceptibility to apomorphine and systems for dopamine (García and Amario, 2001), as well as for γ-aminobutyric acid (GABA) and glutamate (Herman et al., 2004; Kovács et al., 2004) might be important. Of course, observed individual variability might also be due to many factors such as level of aggressiveness (Gerra et al., 1998), body condition, or other physiological variables (Alexander et al., 1993; Moore and Jessop, 2003).

Ultimately, however, this individual variation is likely to be the result of the relative genetic diversity of this population of Djungarian dwarf hamsters (*Phodopus campbelli*) as compared to typical laboratory rodents. Glucocorticoid reactivity shows moderate to high heritability in different animal species (Odeh et al., 2003; Pottinger and Carrick, 1999; Solberg et al., 2003) and thus is affected by the genetic diversity of the study population. Humans also have wide population variation in glucocorticoid concentration influenced by genomic factors (Bartels et al., 2003; Levine et al., 1989) as well as the large variety of developmental and current events that shape their responses (Roy, 2004; Roy et al., 2001). Consequently, the current study both cautions against ignoring individual differences to yield an average population response, and suggests that outbred animal models might be particularly relevant to understanding stress-related pathological conditions. This does not supplant a role for comparison across inbred strains and species, but does suggest that due consideration should be given to the residual variation that remains when an experiment is well-controlled.

Chapter 4

Multiple behavioral parameters fail to predict multiple parameters describing the cortisol response to stress in an outbred hamster (*Phodopus campbelli*)

4.1 ABSTRACT

An outbred species of dwarf hamster (*Phodopus campbelli*) with large between-individual variability in the cortisol response to acute restraint stress was used to assess the association between behavioral phenotype in emotionality-related tests and the cortisol change during an acute restraint stressor. Two selected lines were included to enhance representation of more extreme cortisol phenotypes. A total of 112 hamsters (53 F, 59M) were sequentially exposed to a battery of behavioral tests: open field, elevated plus-maze (not used in analyses), light/dark box, holeboard, resident-intruder, and resistance to capture. Repeated plasma sampling, under home cage anesthesia, 50 min before, and then 10, 60, and 120 min after the onset of a 6 min restraint was then used to quantify cortisol as a physiological measure of the stress response. Cortisol results were transformed into 5 different parameters (initial concentration, average concentration, reactivity, and area under the curve) with different degrees of association. Twenty-two behavior measures were transformed into Z-scores oriented by *a priori* expectations of lower versus higher anxiety, then integrated into four parameters (open field score, hole-board score, light/dark box score, and capture score) and two principal component analyses. Like the cortisol parameters, results differed between individuals, but also differed across measures expected to quantify similar traits. Multiple stepwise linear regression models were then used to explore associations between parameters representing the physiological response to acute restraint stress, and

parameters representing individual behavior. Associations were weak or absent. Results suggest that, particularly in outbred populations, inference from physiology to behavior and from behavior to physiology is likely to be problematic.

4.2 INTRODUCTION

Dynamically increased glucocorticoid concentration is closely associated with stresses such as fear and anxiety (Boissy, 1998; Veenema and Neumann, 2007) and the magnitude of this glucocorticoid response is frequently used to evaluate the impact of a stress upon an individual (Armario, 2006; Mormède et al., 2007). For example, laboratory populations of rats and mice are typically observed in standardized behavioral tests such as an open field or elevated plus-maze, and the combination of behavioral and hormonal responses shown by individuals is then interpreted as a measure of anxiety (Cryan and Holmes, 2005; Finn et al., 2003). Sometimes, the behavioral and the physiological measures are well correlated, but often, the two types of measures lead to divergent interpretations (Ramos and Mormède, 1998; Wahlsten et al., 2003a). Moreover, this divergence is not restricted to animals in laboratory environments (Williams, 2008; Zera et al. 2007).

Two major sources of variation contribute to these interpretation challenges. First, the parameters used to describe the glucocorticoid are not standardized (Fekedulegn et al., 2007; Lee et al., 2007). Reported parameters range from descriptors of the population distribution of concentrations such as the mean or the variance, to integrative parameters such as the area under the curve (AUC), that attempt to describe the overall response, and rate of change parameters such as the slope of the change between samples (Guimont and Wynne-Edwards, 2006; Lasikiewicz et al., 2008; Pruessner et al., 2003). Even these parameters, however, differ in their method of calculation from study to study (Pruessner et al., 2003). Finally, even parameters that co-vary, can lead to fundamentally different interpretations of the results (Fekedulegn et al., 2007; Lee et al., 2007; McGlothlin and Ketterson, 2008).

The second major source of variability is differences in the behavioral measures. Some of this variability results from subtle differences in the behaviors being measured that are sensitive to the precise experimental conditions. Additional variability derives from the use of different strains and breeds of individuals. Still more variability arises when different species are compared, since considerable variability exists across breeds and species in their functional mechanisms of adaptation and responses to environmental challenges (Forkman et al., 2007; Williams, 2008)

The current study was designed to exploit the exceptional individual variability in cortisol responses to a standardized stressor recently described for Djungarian hamsters (*Phodopus campbelli*; Guimont and Wynne-Edwards, 2006), using an extensive battery of standardized behavioral tests, to identify predictive relationships, if any, between behavior and stress responses of individuals.

4.3 METHODS

4.3.1 General methods

4.3.1.1 Animals

All husbandry followed details reported in Guimont and Wynne-Edwards, 2006. Dwarf hamsters (*Phodopus campbelli*) were descendents of an outbred colony maintained at a population size of several hundred individuals, for which standard husbandry precludes pairings closer than second-degree cousins or retention of multiple litters from a single breeding pair. Founding hamsters were imported from the United Kingdom in 1981, then outbred in 1984 with a laboratory stock from Moscow, Russia, and subsequently outbred three times against wild-caught

stock in 1988-90 (Wynne-Edwards, 2003). All hamsters were cared for in accordance with the Canadian Council on Animal Care under Queen's University protocol 041. Photoperiod was 14L:10D with 0000h defined as the middle of the dark phase. Ambient temperature was 18˚C.

In addition to this outbred population, the diversity of cortisol responses to stress was increased by the inclusion of individuals from two derived lines. Bi-directional selection was based on parameters describing individual responses to a standardized, on-back, restraint for 6 minutes (Guimont and Wynne-Edwards, 2006). In generations 1-3, the 5 males and 5 females with the largest cortisol amplitude (line "H") and the equivalent pairings with the lowest cortisol amplitudes (line "L") were selected and bred. Amplitude was calculated as the difference between the concentration in the time point immediately following the end of the on-back stress and the average of the other three time points (prior to stress plus two samples during 2h of recovery). In the third generation, this had resulted in two lines that primarily differed in their cortisol concentration in the initial sample before stress - with the lower amplitude line associated with high initial cortisol concentration and the high amplitude line associated with a low initial concentration. Generations 4 and 5 were then selected based on this divergent criterion. In the 5th generation, "L" comprised 7 males and 12 females, and "H" comprised 22 males and 11 females. All of these animals were tested, as were 30 male and 30 female, age-matched, adults from the parental, outbred, population.

4.3.1.2 Behavioral tests

Behavioral tests were conducted in the same order for each animal starting with the open field and proceeding through elevated plus maze, light/dark box, handling test, holeboard, and intruder aggression test. At least one week elapsed between successive tests for any individual. All animals were 79-101 days of age at the start of behavioral testing and between 160 and 212 days of age during the stress response test. Novel environment and handling tests were conducted

under full-spectrum fluorescent light (~ 350 lx) between 12h30 and 15h30 in order to maximize potential light aversion in this nocturnal species. In contrast, the territorial aggression test was under dim red illumination (~ 15 lx) between 23h00 and 02h00 to optimize the measurement of aggressive component of the behavior. Each apparatus was cleaned with Mikro-Quat® between animals.

4.3.1.3 Open-field

The open-field provides novelty, absence of shelter and landmarks, and bright light (Boissy, 1995). Increase in defecation and locomotor activity in response to the novel open environment (from which escape is prevented by surrounding walls) is typically reported as 'anxiety-related' behavior (Prut and Belzung, 2003; Rushen, 2000), or neophobia in response to the novel environment (Ramos et al., 2003). One animal was placed in a clear Plexiglas™ arena: 25 cm x 25 cm x 14.5 cm (L x W x H) with the floor divided into 25 equal squares. Every 10 seconds for 5 minutes (30 measures), the location of the head was recorded (outer vs inner squares), plus the presence or absence of wall climbing attempts, jumping, urination, grooming, scent marking and thigmotaxis, and the number of fecal pellets, and the reaction to capture at the end of the experiment (see resistance to human capture) were recorded.

4.3.1.4 Holeboard

The holeboard is an open-field with holes through the floor so that head dipping can be used as a measure of exploration distinct from motor activity (File, 2001). The clear Plexiglas™ arena had a black, 40 cm x 40 cm x 13.5 cm, floor containing four holes (3.8 cm diameter; 2.5cm deep). Every 10 seconds for 5 minutes (30 measures) the same measures as the open field plus head dipping and body postures indicating social defeat (the animal is crawling slowly, flattening the body toward the floor) or minimal olfactory trace (abdomen with ventral sebaceous gland

elevated; Wynne-Edwards et al., 1992), and the reaction to capture (see below) at the end of the experiment were recorded.

4.3.1.5 Elevated-plus maze

The elevated plus-maze (EPM) has two (opposite) enclosed and two open arms elevated from the floor. Rats and mice treated with anxiolytic drugs increase the exploration of open arms whereas anxiety-inducing drugs decrease exploration (Lister, 1987; Pellow et al., 1985). The anxiogenic stimulus in this apparatus is the open space rather than height or novelty (Treit et al., 1993). The EPM has been effectively used to study regulation of the HPA axis in mice (Belzung and Griebel, 2001) and is reliable in a wide range of strains, housing conditions, and species, although baseline scores are variable (File et al., 2000). The EPM was made of black Plexiglass (height 31 cm; 4 arms each 30cm long and 6 cm wide; surrounded by a cushioned surface at ground level) and each animal was introduced in the 6 cm^2 center, facing a closed arm. The open arms were covered with fine silica dust to ensure grip for the animals. Moreover, a removable wall (height: 3cm) was fixed along the side of one of the open arms to evaluate possible thigmotaxis. Over 5 minutes, the number of entries into an open arm, number of entries into a closed arm, time spent in open arms, time spent in closed arms, and the time spent in the central square, were recorded. In addition, the presence or absence of head-dipping, and scent marking was recorded in 10 second intervals.

4.3.1.6 Light/Dark box

The light/dark box allows nocturnal rodents to explore a novel environment containing an aversive (open and light) area plus a less aversive (dark and covered) area (Crawley, 1981). The number of transitions from light to dark is correlated with exploratory behavior but not with open field locomotor activity (Crawley, 1981; File et al. 2000). Fewer transitions are interpreted as

anxiety-like behavior although effects are not always consistent across mouse strains or drug treatments (Bourin and Hascoët, 2003). The light/dark box was 44 cm x 19 cm x 30 cm with the light section at ~ 350 lx and the dark section (18 cm x 19 cm) at ~ 35 lx connected by an 8 cm x 7 cm door. Each test lasted 10 minutes to allow comparison of the first and the last 5 minutes, since the presence of an artificial "burrow" can differentiate groups after the first phase of locomotor activity (Blanchard et al., 1994; File et al., 2000). The test started in the light section facing away from the door. The number of crossings from the light to dark side, time spent on each side, latency to leave the light side, and reaction to capture at the end of the experiment were recorded.

4.3.1.7 Resistance to human capture

Capture scores are routinely used to assess domestication (Connor, 1975; Jones et al., 1994; Kalynchuk et al., 1997; Plyusnina and Oskina, 1997; Satterlee and Jones, 1997; Wahlsten et al., 2003b). A 5-point scale derived from Albert and Richmond (1975) was adapted for dwarf hamsters as follows: 0 = easy to pick up, 1 = runs away from hand, 2 = vocalizes in hand, 3 = boxes the hand (upright posture delivering strikes by both paws with eyes closed), 4 = bites or attempts to bite, 5 = bite plus vocalization and/or release of musk from the glands at the openings to the cheek pouches (Wynne-Edwards et al., 1992). The gloved hand slowly approached the hamster immediately after opening the cage lid and then opened and grasped the hamster while allowing an escape forward. Each hamster was independently rated by two observers on two different days.

4.3.1.8 Intruder test

Intraspecies aggression is typically altered by domestication, and is typically interpreted as reduced emotional reactivity (Price, 2002). Proactive individuals initiate an attack whereas reactive individuals flee or respond to attack (Koolhaas et al., 1999). In addition, proactive

animals have a smaller increase in plasma glucocorticoid in response to stress compared to reactive animals (Koolhaas et al., 1999; Schjolden et al., 2005). This test has been used previously for this species in this laboratory (Hume and Wynne-Edwards, 2005; 2006; Wynne-Edwards and Lisk, 1987) and a positive relationship between increases in cortisol and levels of aggression in the intruder test has been reported for the closely-related Siberian hamster (*Phodopus sungorus*; Demas et al., 2004). Each test lasted 10 minutes or until an attack with bite occurred. Control animals served as the age/weight-matched, like-sexed intruders. Variables measured were boxing (see above); attack with bite, flee, and on-back posture.

4.3.2 Integrated behavioral scores

To facilitate integration and comparison across behavioral tests, all continuous behavioral measures were transformed into *Z* scores with a mean of zero and a standard deviation of one. Discrete variables, such as the number of fecal pellets, and ordinal variables (pickup reaction scale after test and resistance to capture test) were tested for nonparametric covariance, regrouped and then integrated into a *Z* score with a distribution equivalent to the parametric measures. Each variable was explicitly polarized with the negative pole indicating an *a priori* expectation of lower anxiety and/or lower aggression (more "*domestic*") whereas the positive pole indicated an *a priori* expectation of higher anxiety and/or higher aggression (more "*wild*").

In addition to these 40 variables, the transformed measures of behavior were averaged within each test to yield an individual score for that test, and then grouped into indices based on covariance and/or *a priori* expectation that they measured similar traits. First, the open-field, holeboard, and light/dark box measures of exploration were combined to yield an exploration index (*Z explo*). Second, resistance to human capture was combined with capture scores from the open-field, holeboard, and light/dark box to form a capture index (*Z cap*). Third, intruder test results, as the sole measures of intraspecific aggression, that could not be combined with other

behavioral measures because results included only the derived lines (parental population served as the intruders, were compiled into an aggression index (*Z IT*). Elevated plus-maze did not contribute to any combined measures (see results). The resulting three behavioral indices were tested in multivariate models.

4.3.3 Cortisol response to the standardized stressor

4.3.3.1 Blood sampling

Blood (150 µl collected into marked 250 µl heparinized Natelson blood collecting tubes, Fisher Scientific) was taken four times from the lateral retro-orbital sinus to yield a time sequence from baseline (t_1 = 50 minutes before the onset of restraint (13h30)) to the immediate post-stress response (t_2 = 10 minutes after the start of a 6 min, on-back restraint) and through recovery (t_3, t_4 = 60 and 120 minutes after the start of restraint). All animals in the room (3-6) were simultaneously held under brief isoflurane (1-chloro-2,2,2-trifluoroethyl difluoro methyl ether) anesthesia for each plasma sample using our established technique for home cage anesthesia that does not result in handling-related changes in cortisol (Guimont and Wynne-Edwards, 2006; Reburn and Wynne-Edwards, 2000). Blood was transferred to a 0.25 ml microcentrifuge tube (Fisher Scientific), centrifuged for 4 minutes at 8000 × *g* and the plasma fraction was stored in 0.2 ml PCR tubes (Fisher Scientific) at –20°C until assayed for cortisol content.

4.3.3.2 Restraint stress

As the standardized restraint (Guimont and Wynne-Edwards, 2006), animals were placed in a clear polyfilm rodent restraint cone (Harvard Apparatus Inc, Holliston, MA), immobilized, inverted, and attached to a Plexiglas™ platform with a 50 mm binder clip. Animals were put in restraint with a 30 second interval between each. After 6 min in the cone, animals were put back

into their modified cages in the same order. All hamsters were then anaesthetized to take the post-stress plasma sample. The on-back position simulates a subordinate social posture, which typically results in immediate urination (Guimont and Wynne-Edwards, 2006).

4.3.3.3 Radioimmunoassay

Cortisol was measured using the ^{125}I Cortisol Coat-A-Count kit from Diagnostic Products Corp., Intermedico, Markham, Ontario). Recommended procedures were followed exactly except that the volume of samples and standards was reduced from 25 µl to 10 µl (Reburn and Wynne-Edwards, 1999; Guimont and Wynne-Edwards, 2006). To minimize inter-assay variability as a source of within-animal error, all samples for every individual were quantified as duplicates within a single assay run. All samples fell within the sensitivity range of the assay (11-247 ng/ml = 83-33% binding). Three human-serum based immunoassay controls (Intermedico, Markham, Ontario) were used to check the reference range of the assay, and two *Phodopus campbelli* blood pools served as internal controls. Intra-assay and inter-assay coefficients of variation for the pooled samples were 9.3% and 15.0% respectively.

4.3.4 Cortisol parameters

After quantification of the cortisol concentration in each of the four repeated samples from each individual, results for each individual were reduced to four *Z* scores (Table 6), describing the individual response to stress in addition with the baseline value. Individual cortisol concentration was represented by the concentration at t_1 (*Z ln* t_1) and the average of the concentration at each time points (t_1, t_2, t_3, and t_4) which were then combined to yield *Z mean CORT*, because successive cortisol measures within an individual over three hours co-vary (Guimont and Wynne-Edwards, 2006). Responses to the stressor were represented by the reactivity (t_2-t_1, *Z CORT Rx*), the proportional reactivity ((t_2-t_1)/t_1, *Z prop CORT Rx*), and the area under the curve relative to

the concentration in the first sample at t_1 ($Z\,AUCi$). This calculation of AUCi yields negative values when, for example, both recovery samples are below the initial sample and the increase in response to the stressor is modest (Guimont and Wynne-Edwards, 2006). These four parameters describing the cortisol response to stress were used in subsequent comparisons to behavioral measures.

4.3.5 Statistical analyses

All statistical analyses were performed with JMP version 7.0 software (SAS Institute, Cary, NC). Significance was taken at $p < 0.05$. All behavioral measures were transformed into *Z* scores (after transformation to achieve normality where required). All *Z* scores were polarized with the negative poles indicating lower anxiety and/or lower aggression phenotype and conversely the positive poles consistent with higher anxiety/aggression phenotypes. Parameters describing the individual cortisol response to stress were also transformed into *Z* scores. Co-variation between primary measures was assessed using product-moment Pearson (r) correlation coefficient or Kendall's rank correlation coefficient (τ) as appropriate. Cortisol data were log-transformed to fit distribution when appropriate. Fits to normal distribution were tested using Shapiro-Wilk *W*. In addition, principal component analyses (PCA) were used to identify cohesive patterns in the behavioral results.

As the primary objective was to evaluate the extent of predictive associations between parameters describing the individual response to a standardized stressor, and the behavior of that individual, multiple, stepwise, linear regression models, including covariates based on previous evidence of association (Guimont and Wynne-Edwards, 2006), were constructed. First, relevant non-behavioral covariates were introduced, and then each cortisol parameter was tested independently.

4.4 RESULTS

As expected based on the selection criteria, inclusion of the three populations (control plus L and H derived lines) broadened the diversity of cortisol responses. For example, populations were clearly differentiated on the basis of AUCi, which assesses an individual's cumulative exposure to additional cortisol throughout the stress and recovery interval (Figure 7). Populations were combined for subsequent analyses.

4.4.1 Hormones

As expected, main effects of acute stress across repeated samples ($F_{(3,100)} = 44.69$, $p < 0.001$) and between sexes ($F_{(1,102)} = 44.04$, $p < 0.001$) were seen (Table 4). For a modest majority of animals (68/110), the highest concentration was the sample immediately following the stressor (t_2). This pattern was consistent in both sexes (males: $F_{(3,53)} = 25.31$; females: $F_{(3,44)} = 21.01$, all $p < 0.001$) with cortisol concentration significantly higher at the second time point (t_2) than in the three other sampling time (t_1, t_3, and t_4). As previously reported, females were higher than males at each time point (all $p < 0.001$; Table 4). The average reactivity (t_2-t_1) did not differ for the two sexes (mean ± SEM) (males: 17.6 ± 2.4 ng/ml, females: 22.2 ± 3.8 ng/ml; $t_{(108)} = 1.04$, $p = 0.30$) as well as the proportional reactivity (males: 46.8 ± 7.9 %, females: 34.4 ± 5.9 %; $t_{(108)} = 1.23$, $p = 0.22$.).

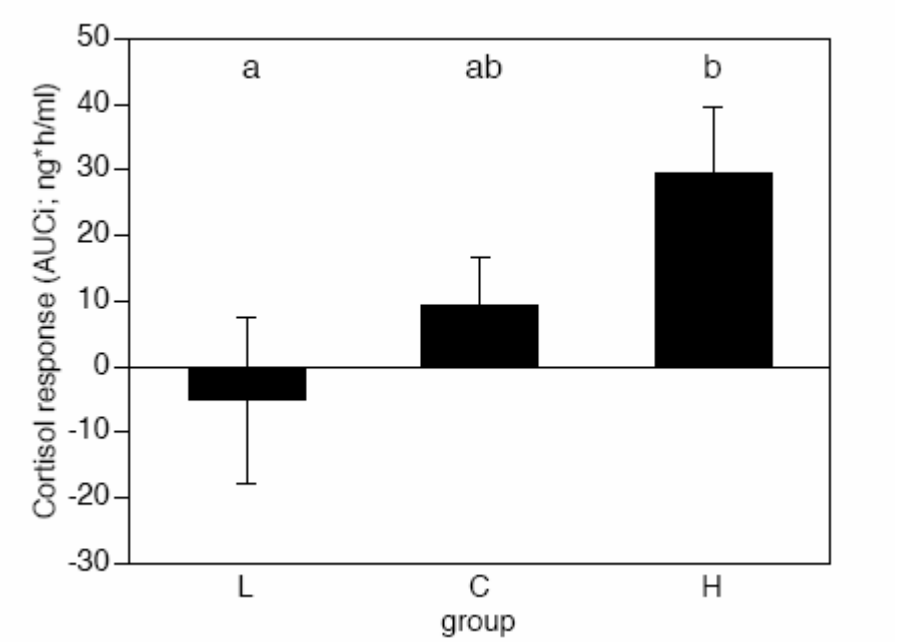

Figure 7. Area under the Curve (AUCi) relative to the initial sample at t_1 for each of the three populations, illustrating the expected differences in individual exposure to additional cortisol throughout the interval of stress and recovery. Negative values indicate that concentrations during the recovery period were lower than the initial sample and more than compensate for the additional cortisol (if any) released in response to the stressor, whereas larger positive values typically indicate that the individual has still not returned to the initial concentration by the last sample. Both the "L" and the "H" derived lines differ significantly from the parental population (different letters indicate $p < 0.05$ for Tukey-Kramer *post hoc* test following one way ANOVA).

Also as expected (Guimont and Wynne-Edwards, 2006), there was considerable variation between individuals in their hormone concentrations. The coefficient of variation (standard deviation/ mean x 100) for the first sample was around 50%, even when males and females were considered separately (Table 4). The inclusion of the three populations also had the expected effect of increasing population variability, since the coefficients of variation in Table 4 are approximately 10% higher than coefficients for the parental population (This study and Guimont and Wynne-Edwards, 2006). Also as expected based on inclusion of the three populations, the coefficient of variation for the area under the curve relative to t_1 (ng cortisol per hour per ml of plasma) was largest of all cortisol parameters, exceeding 400% (Table 5), even though the

parameter was an excellent fit to a normal distribution ($W = 0.99$; $p = 0.84$) and did not differ between the sexes ($t_{(101)} = 0.07$, $p = 0.94$).

Table 4.

Plasma cortisol concentrations (ng/ml).

Sampling time	Sex	Range	*n*	min	max	Mean	SEM	SD	CV %
Stress -50 min	All	197	112	20	217	75.5	4.3	45.1	59.8
(t_1)	Males	180	59	20	200	53.9	3.7	28.3	52.5
	Females	177	53	40	217	100.1	6.7	48.2	48.2
Stress + 10 min	All	197	112	22	219	95.1	4.2	44.1	46.4
(t_2)	Males	196	59	22	218	71.4	3.9	30.2	42.2
	Females	179	53	40	219	122.4	5.9	42.0	34.3
Stress + 60 min	All	221	112	11	232	70.4	4.5	46.6	66.2
(t_3)	Males	114	59	11	125	49.1	3.3	25.2	51.3
	Females	215	53	17	232	95.1	7.5	53.3	56.1
Stress + 120 min	All	231	112	16	247	68.2	4.2	42.9	62.9
(t_4)	Males	93	59	15	108	50.5	3.1	23.3	46.2
	Females	231	53	16	247	88.9	7.3	51.0	57.3

Table 5

Area under the curve with respect to t_1 (AUCi) (ng·h/ml)†.

Sex	Range	*n*	min	max	Mean	SEM	SD	CV %
All	294	112	-135	159	11.9	5.5	55.3	465.0
Males	226	59	-116	110	12.3	6.0	44.5	361.8
Females	294	53	-135	159	11.5	9.7	66.5	578.3

† Negative numbers are typically associated with recovery to a concentration below initial and a small increase following the stressor (see text for details)

After transformation into *Z* scores, parameters describing the individual cortisol response to stress had various levels of correlation (Table 6). In particular, the concentration in the initial sample prior to the stressor ($Z \ln t_1$) was positively associated with the average concentration across all four repeated samples (*Z mean CORT*) and the reactivity (*Z CORT Rx*) was positively associated with the proportional reactivity (*Z prop CORT Rx*) and the AUCi (*Z AUCi*).

Table 6

Pearson product-moment pairwise correlation coefficient (*r*) for cortisol parameters

Cortisol metric	*Z* lnt_1	*Z mean CORT*	*Z CORT Rx*	*Z prop CORT Rx*	*Z AUCi*
Z lnt_1[1]	1.00	0.90	-0.28	-0.56	-0.40
Z mean CORT[2]		1.00	*0.04	-0.22	*0.00
Z CORT Rx[3]			1.00	0.83	0.79
Z prop CORT Rx[4]				1.00	0.70
Z AUCi[5]					1.00

Values shown are for relationships between parameters after Z score transformation (see methods). Details of the calculations for each parameter are: * = not significant, $p > 0.05$.

[1] Z score of the concentration in the initial sample after ln transformation to achieve a normal distribution.

[2] Z score of the average concentration of the four plasma sampling time points after ln transformation to achieve a normal distribution.

[3] Z score of the cortisol reactivity to the stressor (t_2-t_1).

[4] Z score of the proportional cortisol reactivity of the stressor in comparison to first sampling point ((t_2-t_1)/t_1) after square root +1 transformation to achieve a normal distribution.

[5] Z score of the area under the curve in comparison with the increase comparison to first sampling point.

4.4.2 Behavior

The elevated plus maze was not useful as tested with this design. A large majority of individuals (71%) explored the edge and then let themselves drop from the maze (as opposed to falling) within 50 seconds and all individuals had left the maze within 250 seconds. Individuals were replaced on the maze, and all dropped a second time with a shorter latency than the first. Thus, the EPM failed to discriminate between individuals.

Other behavioral tests yielded a broad range of individual variability as expected for this outbred population. For example, 50% of individuals spent 10-20% of their time in the exposed inner sectors of the open field and a similar proportion of individuals spent 20- 29 % of their time head-dipping on the holeboard, although the range of individual behaviors was wide for each measure (Figure 8). However, these two measures, both of which were expected to indicate lower

levels of anxiety at higher values and higher levels of neophobia at lower scores, were not correlated within individuals ($\tau = 0.06$; $p = 0.38$) (Figure 8). In addition, more than 50% of individuals crossed the light-dark boundary in the light-dark box between 10 and 14 times in the course of the 10 minute test (Figure 9) although two individuals failed to make any transitions and one individual crossed more than 35 times. In general, correlations for the same behavior (grooming, rearing, and digging) in different tests were modest or absent (Table 7). However, correlations between the measures contributing to the *Z explo* were significant except between the holeboard and the light/dark box (Table 8). As expected the *Z cap* was not associated with *Z explo*.

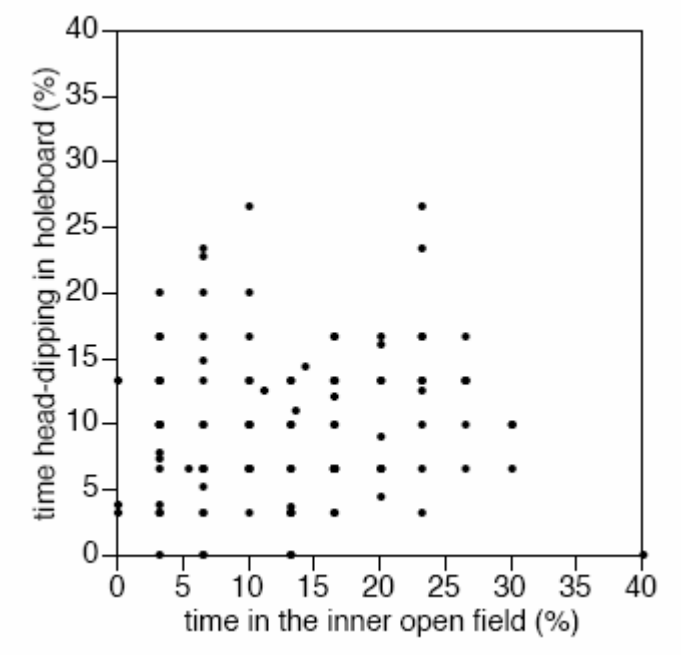

Figure 8. Proportion of the testing time spent in the center (inner sector) of the open field versus proportion of time spent head-dipping in the holeboard for all individuals tested. Note the broad range of individual variability for each measure and the lack of correlation between the two measures, both of which were expected to indicate lower levels of anxiety at higher values and higher levels of neophobia at lower scores.

4.4.3 Principal Component Analysis

In the first PCA, using raw behavioral scores, variables with low frequencies, highly correlated within a test, or non-independent (e.g. inner *versus* outer central area locomotion), were excluded. Two principal components with an eigenvalue higher than 2 were identified. Together they accounted for 37.8% of the variance (Table 9). The variables that positively loaded highly on factor 1 were jumping and rearing in all tests. Factor 1 also loaded moderately for the time in the light section of the light/dark box and negatively for the head-dipping in the holeboard. The second factor loaded positively for grooming and negatively for digging in all tests. It also loaded moderately for the inner locomotion in the open field but not in the holeboard. Thus, factor 1 consisted mainly of behavior related to escaping whereas the second factor was related to general activity level.

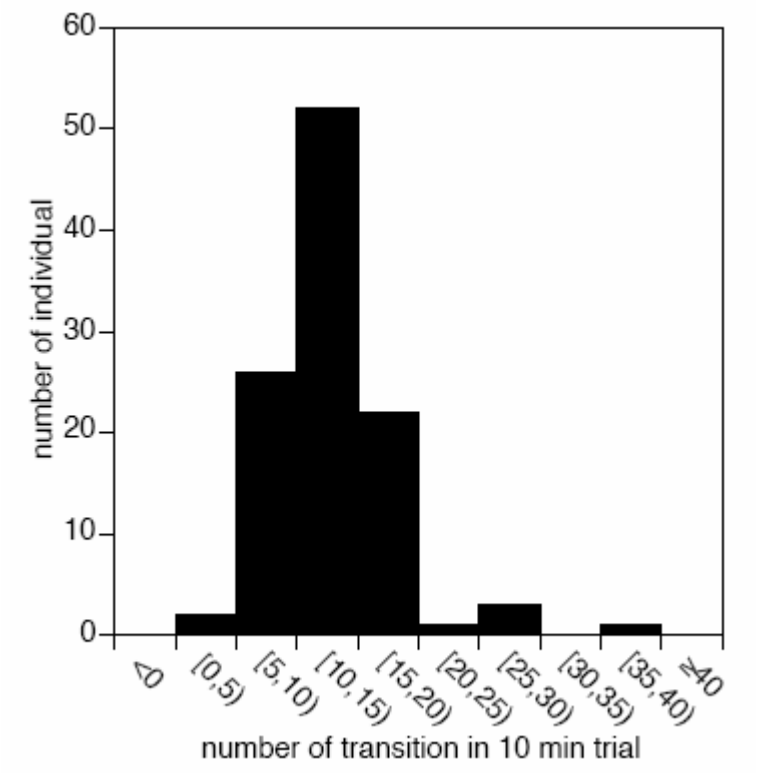

Figure 9. The number of transitions from the light to the dark sector during the 10 minute behavioral test in the light/dark box, showing a strong modal tendency (more than 50% of individuals) to cross between 10 and 14 times with two individuals failing to make any transitions and one individual crossing more than 35 times.

In the second PCA, using the *Z*-scores, two principal components had an eigenvalue higher than 1 and together they accounted for 68.9% of the variance (Table 10). Factor 1 loaded negatively for the three exploration component variables while factor 2 loaded positively for the "reaction to capture" index (*Z Cap*). The *Z HB* also loaded with a similar impact, but opposite direction, in both factors. Thus, the first factor consisted of measurements of exploration and anxiety whereas the second factor was strongly, but not exclusively, related to reaction to capture.

Table 7

Kendall rank correlation coefficient τ of matched behaviors in different tests.

	Activity								
	Grooming			Rearing			Digging		
Apparatus	Open field	Hole board	L/D Box	Open field	Hole board	L/D Box	Open field	Hole board	L/D Box
Open field	1.00	0.30	0.18	1.00	0.39	0.40	1.00	0.25	*0.07
Holeboard		1.00	0.19		1.00	0.30		1.00	0.20
L/D Box			1.00			1.00			1.00

* Correlations non significant, $p > 0.05$.

Table 8

Pearson product-moment pairwise correlation coefficient *r* of transformed behavioural scores.

Behavioral test (transformed)	*Z OF*	*Z HB*	*Z L/D*	*Z Cap*
Z OF	1.00	0.32	0.52	*-0.13
Z HB		1.00	*0.17	*0.09
Z L/D			1.00	*-0.14
Z Cap				1.00

* Correlations non significant, $p > 0.05$.

4.4.4 Stepwise linear regression of behavioral measures against cortisol parameters

At the end of the independent analyses of the cortisol response to stress and the battery of behavioral tests, a wide range of variables were available to be tested for associations between physiological and behavioral measures for each individual. Multiple, stepwise, linear regression with a threshold of 0.10 to enter and 0.10 to leave was used to 'hunt' for strong associations between physiology and behavior. As there were 5 parameters describing the cortisol response to stress, each was tested separately to see if individual variability could be predicted by any of the parameters describing behavior. For each of the five parameters, four different models were constructed against different categories of behavioral measures. In the first and second models,

only the first two principal components were introduced (Model 1 = PCA for raw scores; Model 2 = PCA for Z-scores).

Table 9

Factor analysis (PCA) with eigenvectors and eigenvalues for 15 variables in three behavioural tests.

Items	Factor 1	Factor 2
Eigenvalue	3.66	2.01
Percentage (%)	24.41	13.39
Cumulative Percentage (%)	24.41	37.80
Eigenvectors		
Open field		
inner locomotion	-0.27	**0.46**
jumping	**0.69**	0.08
grooming	-0.33	**0.65**
rearing	**0.72**	-0.07
digging	-0.22	**-0.46**
Holeboard		
inner locomotion	-0.02	0.08
head dipping	**-0.35**	-0.28
grooming	-0.24	**0.64**
rearing	**0.69**	0.04
digging	-0.27	**-0.35**
Light/Dark Box		
time in light	**0.54**	-0.19
jumping	**0.79**	0.02
grooming	-0.03	**0.58**
rearing	**0.85**	-0.07
digging	-0.03	**-0.46**

Boldface = factors greater than 0.35.

Table 10

Factor analysis (PCA) with eigenvectors and eigenvalues for four compound behavioural scores.

Items	Factor 1	Factor 2
Eigenvalue	1.67	1.09
Percentage (%)	41.75	27.19
Cumulative percentage (%)	41.75	68.94
Eigenvectors		
Z OF	**-0.84**	0.02
Z HB	**-0.58**	**0.51**
Z L/D	**-0.77**	-0.23
Z Cap	0.15	**0.88**

Boldface = factors greater than 0.35.

In the third model, the four compound behavioral scores (*Z OF, Z HB, Z L/D, Z Cap*) were introduced. In the fourth model, all 22 behavioral scores (Z-scores) for each animal were simultaneously introduced. As expected, models incorporating absolute cortisol parameters ($Z \ln t_1$ and *Z mean CORT*) as dependent variables, identified sex as a significant factor ($p < 0.05$) so that those models were run separately for each sex. Thus, 28 stepwise models, involving a total of 30 potential measures of behavior and five measures of the cortisol response, were run. The overall result was a resounding lack of strong association, in spite of the statistical expectation that spurious associations would be identified because of the large number of comparisons.

None of the many models identified consistent, strong, association between physiology and behavior. In the first model, *Z mean CORT* was significantly associated with the 2nd factor of the PCA (representing general activity) in females (adjusted $R^2 = 0.14$, $F_{(1,45)} = 8.67$, $p < 0.01$). However, none of the other four cortisol parameters were associated with the PCA components from the raw data. In the second model, *Z CORT Rx* was associated with the second PCA component from the Z-score analysis in females (adjusted $R^2 = 0.10$, $F_{(1,49)} = 5.74$, $p < 0.05$). However, none of the other four cortisol parameters were associated with the PCA components from the Z-score data. In the third model, with integrated behavioral scores, *Z CORT Rx* was also associated with *Z L/D* and *Z Cap*, but again the predictive value was low (adjusted $R^2 = 0.09$). Finally, the fourth model, with 22 Z-scores for different behavioral measures, identified an association restricted to males between digging behavior in the light/dark box and the associated variables of $Z \ln t_1$ (adjusted $R^2 = 0.06$, $F_{(1,57)} = 4.88$, $p < 0.05$) and *Z mean CORT* (adjusted $R^2 = 0.10$, $F_{(1,57)} = 7.75$, $p < 0.01$).

4.5 DISCUSSION

As expected, Djungarian dwarf hamsters (*Phodopus campbelli*) showed wide individual variation in their cortisol responses to a standardized, acute, on-back restraint. Also as expected, the behavioral responses of individuals varied widely across individuals and within individuals over different behavioral tests. However, despite the exceptional diversity of behavioral measures and the calculation of multiple parameters describing the cortisol dynamics within individuals, there was exceptionally little evidence of association between behavioral and physiological measures for the same individual. These results suggest that neither a within-individual change in plasma nor a battery of behavioral tests is an adequate proxy for the stress response of individuals.

It is already well established that the sources of variation in glucocorticoid concentration and behavior are diverse and complex (Mormède, 2007; Sapolsky et al., 2000). Glucocorticoid concentration variation can be found in other outbred species (see Williams, 2008 for a review) and wide variation is also observed in behavioral responses to stress related tests (Forkman et al., 2007). On the other hand, many studies in laboratory animals provide convincing evidence of behavioral consistency in individual responses to different adverse situations (Brush et al., 1985; Goddard and Beilharz, 1984). Thus, individuals able to display stable behavioral traits over time such as aggressiveness, reaction to novelty, willingness to take risk and exploration have been reported in many species (see Réale et al., 2007 for a review) and selection experiments based on these behaviors have been reliably linked to changes in hormonal responses to stress (Landgraf and Wigger, 2002; Trut, 1999; van Oortmerssen and Bakker, 1981). However, the current results suggest that it is probable that behavioral and physiological responses to a standardized stressor are less tightly linked than the majority of studies assume them to be.

We did not detect any strong linear correlation between behavioral response to emotional stress and cortisol increase after physical stress. Similarly, a recent study showed that the increase or decrease of corticosterone in male rats was not proportional to the locomotion and anxiety related behavior elicited by the open field test and the elevated plus-maze (Mikics et al., 2005). We also found that some individuals responded differently to different tests expected to measure similar aspects of individual personalities. For example, the three exploratory tests segregated from each other in the principal components analysis and showed weak association across the same behavioral measures. We also found that integrated measures of exploratory behavior separated clearly from integrated measures of escaping and handling behaviors in the principal components analysis. These results suggest that anxiety in novel environments and the reaction to capture/ response to an intruder are independent. These axes could be similar to the ones proposed by Koolhaas and collaborators (2007) that are defined as the emotional axis and coping style axis respectively.

On the other hand, the cortisol response was measured relative to a physical restraint in a socially subordinate position, which was not necessarily analogous to any of the behavioral stresses that the animals faced. Naumenko and colleagues (1989) have shown that a rat's behavior toward emotional stress (reaction to capture by human hand test) was not correlated to its corticosterone reactivity following a physical stress (tail cutting). The differential affinity of two corticosteroid receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) might contribute to the diversity and independence of behavioral and physiological responses. MR has a tenfold higher affinity for glucocorticoid than GR (de Kloet et al., 2008). Theoretically then, emotional responses might be less intense than physical responses, binding only the MR whereas physical stress might bind both the MR and the GR (de Kloet et al., 2008; Korte et al., 2005; 2007). For example, by blocking the MR with RU486, Smythe and collaborators (1997) reduced

anxiety in rats tested in the light/dark box. On the other hand, the activation of MR and GR in mice reduced their anxiety behavior in the holeboard (Brinks et al., 2007). Thus, if it were possible to measure the stress response of each individual relative to each behavioral test, then strong associations might emerge. In practice, however, this is unlikely as accepted methodologies emphasize multiple behavioral tests and avoid multiple bloods sampling because of the effects of learning (Herrero et al., 2006; Márquez et al., 2006).

This study, therefore, adds to concerns that, particularly in diverse populations such as humans, inference from physiology to behavior and from behavior to physiology is likely to be problematic. The relationship between glucocorticoid dynamics, emotional behavior, and physical stress are not simple, nor do they generalize from one situation to another. Well designed studies, with multiple integrative measures, are therefore needed to establish if, and how, such complex approaches can guide practical approaches to mitigating, and medicating, the stress response.

Chapter 5

General Discussion

The first goal of this thesis was to evaluate the homogeneity of individual variation in the HPA axis reaction curve in an outbred laboratory population of Djungarian dwarf hamster (*Phodopus campbelli*). In spite of a high degree of experimental control to ensure that the acute, on-back restraint was uniform for each individual, the experiments presented in Chapter 3 showed exceptionally large individual variation. This variability was not restricted to a single parameter describing the cortisol response to stress. Neither cortisol concentration, nor reactivity, nor area under the curve explained or reduced this variability. Cortisol responses to acute on-back restraint could not be grouped by sex, age, or housing conditions. Thus, the hypothesis of homogeneity across individuals was rejected.

The second goal of this thesis was to explore the association of this individual endocrine variation with individual emotion-related behaviour. Although many behavioural tests were conducted, and multiple statistical approaches were combined to identify underlying patterns of behaviour within individuals, there was no support for the hypothesis that one or more of the parameters describing the physiological response to stress would be predictive of one or more of the behavioural parameters for the same individual. Thus, the hypothesis was rejected. In this outbred, wild-derived species, the cortisol response to a standardized, acute, on-back restraint stressor is not predicted by behavioural responses to novel environments expected to be stressful, social interactions with an intruder, or even overall measures of physical activity. This contrasts with the conclusions of previous studies using more genetically homogenous study populations (Dellu et al., 1996; Kabbaj et al., 2000; Landgraf and Wigger, 2003; Touma et al., 2008).

Rejection of both of these working hypotheses raises three primary issues that are not resolved by the current data. First, the role of heritable genetic factors in the responses of individuals is not known. Second, the relative 'value', precision, and reliability of physiological *versus* behavioural measures for the subjective experiences of the animals remains unknown. Finally, the generality of widely-used tests to assess stress and distress in diverse species might be of limited value in these hamsters.

5.1 Individual variation of HPA axis as an opportunity in evolutionary endocrinology

The large variation observed in the stress response observed in *P. campbelli* would be ideal for studies of the genetic basis for different components of the physiological response to stress. Artificial selection of genetically differentiated populations could identify the underlying causes of phenotypic divergence and correlations (Zera et al., 2007). In fact, previous reports on fish, birds, and mammals with large variation in plasma glucocorticoid concentrations in response to acute stress (e.g. Pottinger and Carrick, 2001; Sinkle and Ditchfield, 2002; Odeh et al., 2003; Touma et al., 2008) have shown significant heritabilities in glucocorticoid reactivity between pre- and post-stress titers. Those data suggest a significant genetic contribution to HPA axis response would be expected. However, such artificial selection experiments are rare because of the time and expense involved in creating and maintaining parallel selected lines for many generations. A rare example is the recent endocrine selection of CD-1 strain mice by Touma and collaborators (2008). Similar experiments with more variable stocks such as *P. campbelli*, would also benefit form the large reproductive capacity and short generation time of small bodied rodents, and would increase our understanding of microevolutionary adaptation and related fitness components.

Multiple examples of microevolution that put pressure on the HPA axis would be expected. Domestication, for example, changes HPA axis dynamic development by interacting with sexual maturation (Shishkina et al., 1993; Trut, 1999) and the serotonergic system (Naumenko et al., 1989; Popova, 2006). Glucocorticoid dynamic changes might also be relevant in animal welfare issues where they could be coupled with other physiological variables such as ACTH dynamics and heart rate to assess animal well-being (Veissier and Boissy, 2007; Mormède, 2007). The same also goes for the stress in reintroduction in conservation genetics (Cockrem, 2005; Frankham, 2005; 2008). Understanding inter-individual variation of HPA axis regulation is also potentially important as part of the explanation for differential immune responses (Koolhaas, 2008; Redei, 2008), and vulnerability to affective disorders such as depression (Touma et al., 2008). Therefore further experiments aiming to understand individual variation in emotional stress reactions should have widespread impact.

5.2 Physiological versus behavioural approaches

Although the current results do not support an association between physiological and behavioural approaches to assessment of animal stress responses, they are not sufficient to reject such an association. The data in this thesis do not assess the repeatability of either the physiological or the behavioural responses of individuals measured repeatedly on the same task. Thus, it remains possible that the single episode that was measured was an inadequate representation of the individual's stress response. Second, the stress that was applied to assess the physiological response to stress might have had a different salience for the animal, and thus a different evoked stress, than the array of behavioural tests.

On the other hand, the data do represent a wide variety of measures, and of statistical approaches, that were likely to reveal an association if that association were robust. Thus, other

interpretations are also possible. First, it is possible that the dwarf hamsters do not have a modulating effect connecting the HPA axis to anxiety-related behavior (Veenema and Neumann, 2007). Nevertheless, HPA axis responsiveness is consistent across different behavioural tests within individuals, at least in domestic pigs when ACTH is the parameter of interest (Zhang et al., 1992). This is also true in Sprague-Dawley rats for corticosterone (Márquez et al., 2005). However, even in the Sprague-Dawley rats, no relationship between resting and stress levels of corticosterone concentration was observed. This echoes the current results.

Also of potential importance to the interpretation of these results is the validity of the assumption that associations would be linear between parameters for individuals. Both high and low HPA axis activity can be associated with excessive aggression, perhaps due to the fact that faulty regulation of HPA axis hormones might contribute to the escalation of violent behavior under stressful conditions (Kruk et al., 2004; Haller et al., 2006). Some research suggests that the corticotrophin-releasing factor receptor type 1 (CRF-R_1) has an important role in this modulation (e.g. Smith et al., 1998; Timpl et al., 1998), as well as galanin (Tortorella et al., 2007), serotonin (Summers and Winberg, 2006), and secretagogue receptors of GABA, MR and growth hormone (GH) (Giordano et al., 2006). The analytical methods used in the current study would not identify complex relationships, such as a u-shaped relationship, that might persist to link the physiological and behavioural measures.

Finally, it remains possible that the HPA axis in dwarf hamsters is primarily variable, from individual to individual, in a different component of the HPA axis pathway. For example rats and mice are sensitive to both the CRF agonist receptors and the 5-HT_{1A} receptor agonist in the open field response (Prut and Belzung, 2003) yet only the 5-HT_{1A} receptor is strongly related to aggressiveness and coping style in mice selected for short and long attack latencies (Veenema et al., 2005). Thus, any comprehensive model must incorporate all of the feedback pathways

modulating the effects of the hormone changes on the intracellular physiology of the stressed individual.

5.3 Are dwarf hamsters 'eccentric' outliers?

Dwarf hamsters did not respond to the standardized behavioral tests in the same way as most strains of mice and rats do. In one of the most widely used measures of anxiety-like behavior, the elevated plus maze (EPM), most of the hamsters leapt from the open arms within less than a minute. In the open field and holeboard tests, hamsters engaged in vigorous locomotor activity directed towards escaping over or under the walls of the apparatus instead of showing exploratory behaviour on the floor. In the light/dark box, animals spent time in both sections and crossed between sections, on average, more than once per minute during the test.

Direct comparisons of our results with the ones from other species are not possible because behavioural data are very sensitive to the exact conditions of the test (File et al., 2000; Belzung and Griebel, 2001; Finn et al., 2003; Blanchard et al., 2003). Nevertheless, rodents like the Wistar rats are clearly distinct from this pattern. Behaviour on the EPM was successfully used as the basis of the selection of Wistar rats for high anxiety-related (HAB) and low anxiety-related behavior (LAB) (Landgraf and Wigger, 2002). HAB and LAB rats differ by open arm entries and time spent in the open arm, but not in total entries or closed arm entries. They also differ in "exploration" (rearings, head dips and latency hole) in a holeboard test (Ohl et al., 2001). LAB rats also have higher inter-male aggression (Veenema et al., 2006), lower HPA reactivity to non-social stress (Salomé et al., 2004), and show a more proactive coping style (Bosch et al., 2005; Veenema et al., 2006). Similarly, mice (*Mus musculus domesticus*) selected for short (SAL) and long attack latency (LAL) (van Oortmerssen and Bakker, 1981) also display a difference in intruder-test where SAL are more proactive and LAL more reactive (Veenema et al., 2004).

Those mice strains also differed in the number of open arm entries as well as total entries into an arm in the EPM (Veenema et al., 2003). In both species, therefore, even strong selection did not result in animals that jump quickly from the EPM, as was universally characteristic of *P. campbelli*.

The current results also suggest that the dwarf hamsters might lack the neophobic response that many of these behavioural tests are designed to exploit. Wild *Phodopus campbelli* males and females also tend to approach novelty in their environment rather than avoid it (Wynne-Edwards, 2003). In nature, and without habituation to humans, dwarf hamsters approach observers seated outside their burrow, enter backpacks to forage, and tolerate extraordinary proximity (Wynne-Edwards, 1995; 2003). Both males and females also readily consume fresh placenta although placental tissue typically evoke a neophobic rejection in all mammals except parturient females (Gregg and Wynne-Edwards, 2005; 2006).

These dwarf hamsters also show less aversion towards the light side of the light/dark box than laboratory rodents previously studied (Hascoët et al., 2001). This is also consistent with behaviour in the wild, as these hamsters routinely emerge from their burrow before complete darkness and return to their burrows after first light (Wynne-Edwards et al., 1999). In contrast, their closest living relative, the Siberian dwarf hamster (*Phodopus sungorus*), restricts its above-ground activity to complete darkness (Wynne-Edwards et al., 1999). This characteristic could probably explain in part the discrepancies between *P. campbelli* and *P. sungorus* previously reported in the open field (Prendergast and Nelson, 2005; Pyter and Nelson, 2006).

Of course, in other respects, the dwarf hamsters were similar to mice. Like mice (Wahlsten et al., 2003b), the majority of animals did not respond with fear or defensive aggression to cage opening, approach of the gloved hand, or capture. Resident animals attacked an age-matched,

same-sex, intruder (Hume and Wynne-Edwards, 2005; 2006) and *P. campbelli* males were more aggressive than females (Wynne-Edwards and Lisk, 1987).

Thus, it is also possible to interpret the current results as 'eccentric' responses characteristic of a mammalian species subject to extreme selection in an exceptionally harsh environment (Wynne-Edwards, 2003). *Phodopus campbelli* adults might just be insensitive to anxiolytic test situations relative to laboratory strains of mice and rats (Finn et al., 2003). Their natural ecological niche might have selected for a willingness to approach and exploit novel resources and events, even if they might be hazardous, because the potential benefit from additional resources outweighs the risk. Their habitat in Central Asia is harsh. Annual rainfall is low and unpredictable, the winters are extremely cold and lack insulating snow cover, and population densities are very low (Wynne-Edwards et al., 1992; Wynne-Edwards 1998; 2003).

5.4 Conclusion

The first comprehensive research effort directed at assessing the extent of individual variability, and the associations between physiological and behavioural measures of that variability, in a wild-derived, outbred, small-bodied rodent has yielded results that are difficult to interpret. Individual variability was unexpectedly large and was not explained by obvious co-variates including sex and age. Individuals were equally variable in their responses to a wide variety of behavioural situations intended to assess neophobia and emotionality. However, there was little, or no, correlation between physiological and behavioural measures of coping strategies in response to stress. Thus, the results suggest the possibility that response to a socially relevant, acute, restraint stressor is dissociated from 'psychological' responses to novelty stresses as expressed through behaviour. Although it remains possible that these results are restricted to the specific case of dwarf hamsters, it remains likely that they are illuminating a larger problem that

results from the generalization of results obtained on inbred, homogeneous, populations to natural populations of animals and humans.

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