Water deprivation induces appetite and alters metabolic strategy in Notomys alexis: unique mechanisms for water production in the desert

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Like many desert animals, the spinifex hopping mouse, Notomys alexis, can maintain water balance without drinking water. The role of the kidney in producing a small volume of highly concentrated urine has been well-documented, but little is known about the physiological mechanisms underpinning the metabolic production of water to offset obligatory water loss. In Notomys, we found that water deprivation (WD) induced a sustained high food intake that exceeded the pre-deprivation level, which was driven by parallel changes in plasma leptin and ghrelin and the expression of orexigenic and anorectic neuropeptide genes in the hypothalamus; these changed in a direction that would stimulate appetite. As the period of WD was prolonged, body fat disappeared but body mass increased gradually, which was attributed to hepatic glycogen storage. Switching metabolic strategy from lipids to carbohydrates would enhance metabolic water production per oxygen molecule, thus providing a mechanism to minimize respiratory water loss. The changes observed in appetite control and metabolic strategy in Notomys were absent or less prominent in laboratory mice. This study reveals novel mechanisms for appetite regulation and energy metabolism that could be essential for desert rodents to survive in xeric environments.

Keywords: xeric adaptation; appetite regulation; brain-gut axis; energy metabolism; oxidation water

1. INTRODUCTION

The amazing ability of desert animals to cope with extreme heat and aridity has fascinated many researchers since the beginning of the last century, and there has been significant progress in understanding the ecology and physiology of desert animals [1–3]. The spinifex hopping mouse, Notomys alexis, is one such animal that thrives in the Australian desert [4–8]. In the laboratory, Notomys can maintain normal water balance (plasma osmolality and blood volume) during total water deprivation (WD) if fed on dry seeds [7,8]. Their xeric adaptability is even greater than the well-studied kangaroo rat (Dipodomys spp.) of similar size, in which WD changes body fluid parameters to some extent [9]. Many studies have shown that Notomys have an extraordinary ability to concentrate urine up to 9370 mOsm [4,6], reduce faecal water content to less than 50 per cent [4], and reduce respiratory water loss by a nasal cooling system [10] and co-habitation in burrows [5]. However, the obligatory water loss by respiration and urine production must be balanced by water gain, which in granivorous rodents is mostly from metabolic (oxidative) water [1,2].

Among substrates for oxidation, lipids (fat) produce twice the amount of oxidation water than carbohydrates (starch) per gram, but starch produces 20 per cent more water than fat per kilocalorie because of a greater demand for oxygen for fat metabolism [1]. Thus, starch is a better substrate than fat for water production in a dry environment, where respiratory water loss needs to be minimized. Protein produces the least amount of oxidation water per gram and necessitates renal water loss to excrete nitrogenous metabolites. Thus, protein oxidation results in a net metabolic water loss even in a humid environment. Despite the importance of metabolic water in desert animals, studies on the mechanisms of its regulation are limited [1–3]. There are studies that show desert rodents can select seeds containing more moisture and less protein for food [3]. However, there has, to our knowledge, been no research on how they change their food intake strategy to increase metabolic water production or how they change metabolic strategy to enhance the efficiency for metabolic water production when exposed to dehydration.

In relation to obesity and metabolic syndrome that are serious public health challenges in the twenty-first century, regulation of appetite has been a target of intensive research in basic and clinical medicine [11]. These studies have unveiled important interactions between and within the central and the peripheral regulatory system via various circulating hormones and neuropeptides [12,13]. Pancreatic and gastrointestinal hormones have been shown to play pivotal roles in

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appetite regulation, of which ghrelin secreted from the stomach is the only orexigenic hormone [14]. Insulin and other gut hormones are important anorectic hormones, but leptin, a product of the obese gene secreted from the adipose tissue, appears to be a primary hormone that counters over-eating [15]. These circulating hormones act on the neurons in the arcuate nucleus (ARC) of the hypothalamus, to which they have access via the median eminence that lacks the blood-brain barrier [13,14]. The ARC neurons contain orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) or anorectic proopiomelanocortin (POMC)/cocaïne- and amphetamine-regulated transcript (CART). POMC is a precursor of the major anorectic neuropeptide, α-melanocyte-stimulating hormone (α-MSH) [16]. They send their axons to neurons in the lateral hypothalamic area (LHA) containing orexigenic melanin-concentrating hormone (MCH) or orexin (ORX) and to neurons in the paraventricular nucleus (PVN) containing anorectic corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and oxytocin (OXY). These peripheral and central signalling molecules work in concert to regulate food intake [12].

It is reasonable to assume that water-deprived desert animals increase food intake to obtain more substrates for metabolic water production, although WD usually suppresses feeding in most animals studied [17]. In this study, we examined how WD affected food intake and its control, which underpins the metabolic strategy of Notomys required for maintaining water balance. WD induced a biphasic appetite response in which an initial hypophagia and body mass started to increase between day 7 and 12 of WD. These time points were chosen following the 28 day experiment, as food intake increased between day 3 and 7 and body mass started to increase between day 7 and 12. Two separate sets of experiments were performed with a combination of Notomys and Mus of Balb-C or Swiss strain. After each WD experiment, mice were anaesthetized by halothane inhalation, and blood was collected by cardiac puncture into a chilled syringe-containing inhibitors (0.05 M 1,10-phenanthroline, 0.225 M potassium Ethylenediamine tetraacid (EDTA) and 0.1 trypsin inhibitory unit aprotinin) [18]. Plasma was separated by centrifugation for measurements of glucose, leptin, ghrelin and corticosterone concentrations. The brain, liver and skeletal muscle were then excised, frozen immediately in liquid nitrogen, and stored at −80°C until use. The brain was used for in situ hybridization of neuropeptides, and the liver and skeletal muscle were used for cDNA cloning and real-time PCR of glycogen synthase transcripts. The stomach and adipose tissue were also collected from Notomys for cDNA cloning of ghrelin and leptin, respectively (electronic supplementary material, figure S3). The procedures for cDNA cloning have been described previously [19].

Plasma ghrelin concentration was determined by a radioimmunoassay that recognizes the C-terminus of rat/mouse ghrelin [20]. Plasma leptin concentration was measured by an ELISA kit for mouse leptin (IBL Co. Ltd., Gunma, Japan). The intra-assay coefficient of variation was 7.5 per cent (n = 4). Notomys ghrelin and leptin sequence exhibited 100 and 95.2 per cent identity to the Mus peptides, respectively (electronic supplementary material, figure S3). Plasma concentration of corticosterone, rodent glucocorticoid, was measured by an EIA kit (YK240, Yanaihara Institute, Inc., Shizuoka, Japan). The intra-assay coefficient of variation was 5.5 per cent (n = 6). Plasma glucose concentration was determined by a glucose CII test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma Na⁺ concentration was determined by an atomic absorption spectrophotometer.
All plasma parameters were measured in duplicate. In addition, body fat content was determined by dual energy X-ray absorptiometry using a Norland pDEXA forearm densitometer (Inderlec Medical Systems, Baulkham Hills, Australia).

Semi-quantitative in situ hybridization was performed using oligodeoxynucleotide probes designed for mRNAs of mice (electronic supplementary material, table S2) based on the method described previously [21]. These probes hybridized well with the mRNAs encoding Notomys peptides. Eight sets of four serial sections of 12 μm were cut per animal and were used for quantification of NPY, POMC in the ARC and of CRH, TRH and OXY in the PVN, while 16 sets of eight serial sections were used for quantification of ORX and MCH in the LHA because of a larger size of this area.

Measurement of metabolic parameters after water deprivation

Tissue glycogen content was measured by the phenol-sulfurous acid method [22], which was modified for this study. The intra-assay coefficient of variation using the tissue from the same mouse liver was 10.5 per cent (n = 3).

Glycogen synthesis activity was assessed by glycogen synthase, which is a rate-limiting enzyme for glycogenesis and its gene expression responds to food intake [23]. To examine the time-course change in the expression of glycogen synthase gene in the liver, quantitative real-time PCR was performed using Power SYBR Green RNA-to-C1-Step Kit (Applied Biosystems, Foster City, USA) using gene-specific primers (electronic supplementary material, table S2). The β-actin gene was used as an internal control. The identical sequence between Notomys and Mus glycogen synthase 2 or β-actin mRNA was used for real-time PCR.

Statistics

All data are presented as means ± s.e.m. Time-course changes in food intake and body weight during 28 day WD was compared with controls by two-way ANOVA, and the pattern of the changes during WD was compared with non-WD controls and between Notomys and Mus by a Bartlett test. Changes in measured parameters at 3, 7 and 12 days of WD were compared with the values before WD (day 0) by the two-tailed Dunnett test. If the normal distribution was not demonstrable, non-parametric methods were applied.

3. RESULTS

(a) Changes in food intake and body mass after water deprivation

Notomys decreased food intake linearly for 5 days after WD and then food intake increased abruptly and exceeded that of water-replete mice after 12 days (figure 1a). The intake for 28 days was not statistically different between WD and non-WD mice, but the pattern after WD was significantly different between the two groups. Although less prominent, body mass also decreased gradually for 10 days and then increased slowly towards the pre-WD level (figure 1b). By contrast, Mus decreased food intake and body mass during WD, and these parameters remained lower than that of water-replete mice after 12 days (figure 1c,d). The decreased food intake was significantly different from that of controls, but the pattern of the change was not different. Thus, there is clearly a distinct response to WD between Notomys and Mus, with dynamic changes in food intake and body weight observed in Notomys.

(b) Changes in body fat and plasma hormone concentrations after water deprivation

As arousal of appetite was apparent in Notomys, we compared the status of the appetite control systems between the two mouse species. Two Mus strains were used in this study, but both strains exhibited similar responses.
to WD in terms of food intake and body mass changes. Therefore, only data for the Swiss strain are shown here as all plasma parameters could be measured in this strain. The \textit{in situ} hybridization data of the Balb-C strain are shown in the electronic supplementary material, figure S2. The biphasic pattern of food intake in \textit{Notomys}, but not in \textit{Mus}, during 12 day WD was also observed in this series of experiments (data not shown). Body mass tended to increase after more than 10 days of WD in \textit{Notomys} (figure 2a) as in figure 1, but body fat decreased linearly and became undetectable after 12 days (figure 2b). Similar to the body fat, plasma leptin concentration decreased linearly (figure 2c). The decrease in body mass at 7 days of WD may be attributable to the loss of body fat. By contrast, plasma ghrelin concentration showed a biphasic change and the level was higher than pre-WD level after 12 days in \textit{Notomys} (figure 2d). In \textit{Mus}, body mass decreased gradually over 12 days (figure 2f), but body fat increased linearly during WD (figure 2g), which are distinct from the changes in \textit{Notomys}. The increase in body fat was not significant during 12 days of WD. Interestingly, body fat content of water-replete \textit{Notomys} was greater than five-fold higher than that of \textit{Mus}. Plasma ghrelin and leptin concentration did not change after WD in \textit{Mus} (figure 2h,i). Plasma Na\textsuperscript{+} concentration did not change during WD in both \textit{Notomys} and \textit{Mus} (figure 2e,f).

\textbf{(c) Changes in hypothalamic neuropeptide genes after water deprivation}

Consistent with the orexigenic stimuli from the periphery, semi-quantitative \textit{in situ} hybridization showed that in the
ARC of Notomys, expression of the orexigenic NPY gene was enhanced linearly (figure 3a), while that of the anorectic POMC gene was suppressed (figure 3d) in the ARC of Notomys after WD. In addition, WD increased the mRNA expression of orexigenic ORX and MCH genes in the LHA (figure 3b,c) and decreased the mRNA expression of anorectic CRH gene in the PVN (figure 3e). However, the mRNA expression of the anorectic TRH and OXY genes was not suppressed in the PVN during WD in Notomys (figure 3f,g). In Mus, the changes in brain neuropeptide mRNA expression generally favoured appetite stimulation except for ORX, TRH and OXY (figure 3h–n), which reflects the suppressed but persistent food intake of this species during WD. However, the changes of such genes for appetite stimulation were clearly more obvious in Notomys than in Mus.

(d) Changes in metabolic strategy after water deprivation

In Notomys, body fat disappeared quickly during WD (figure 2b), but body mass started to increase after 12 days (figures 1 and 2a); this indicates the accumulation of nutrients other than fat. Thus, we examined the changes in glycogen content in the liver and skeletal muscle during WD. Consistent with the gradual increase in body mass in the later phase of WD, glycogen content in the liver increased linearly after an initial decrease for 3 days in Notomys (figure 4a). The muscle glycogen level changed in a similar pattern but its concentration was 1/30 that of the liver (figure 4b). The hepatic glycogen synthase gene expression decreased for 7 days after WD, but increased suddenly to the pre-WD level after 12 days (figure 4c). Plasma glucose concentration gradually decreased during the 12 days of WD (figure 4d) even with increased food intake after 5 days, suggesting enhanced glycolysis and glycogenesis. As a possible trigger for glucose utilization, plasma corticosterone concentration increased gradually after WD in Notomys (figure 4e). In contrast to Notomys, hepatic glycogen content decreased linearly in Mus during WD, and the decrease was significant after 12 days (figure 4f). The muscle glycogen content also decreased after WD (figure 4g). Hepatic glycogen synthase gene transcript decreased slightly but significantly after 3 days of WD in Mus (figure 4h). Plasma glucose and corticosterone concentration did not show obvious changes during WD in Mus (figure 4i,j).

4. DISCUSSION

It was believed that desert rodents acquire water by oxidation of nutrients stored in the body when they encounter water shortage to compensate for obligatory water loss by respiration, and in urine and faeces [1–3]. It was also assumed that they may increase food intake to supply more substrates for oxidation as water deficiency is prolonged. However, attempts to measure food intake in desert rodents have not been successful because they instinctively disturb seeds and mix them with the substrate (sand, etc.). Thus, we initially developed a feeding station that enabled us to measure food intake accurately. Using this feeding system, we found that water-deprived Notomys showed a distinct biphasic feeding pattern during WD; there was an initial decrease for 5 days and a subsequent abrupt increase over the remaining WD period. The WD-induced food intake will increase the available substrates for metabolic water production and, in combination with the mechanisms to decrease water loss, contribute to the maintenance of water balance in desiccative environments. This is, to our knowledge, the first evidence showing a unique appetite control mechanism in desert rodents as a physiological adaptation to aridity. The initial hypophagia for 5 days of WD is consistent with previous studies showing that many mammals reduce feeding in response to WD [17], which is attributed to factors such as the behavioural linking of eating and drinking, a dry mouth and the possible effect of osmoreceptor suppression on feeding [24]. Unexpectedly, we also found that laboratory mice (Mus) used as non-xeric controls can withstand 28 days of WD, although they decreased food intake and body mass gradually over the period of WD, in contrast to Notomys. The ability of Mus to tolerate WD may be owing to the low protein and salt content of millet seed and the evolutionary origin of this species in the arid area of the Far East prior to being established as a laboratory animal [25].

In this study, we also showed that major appetite stimulating (ghrelin) and suppressing (leptin) hormones found in the plasma work in concert to increase food intake when Notomys are subjected to prolonged WD. Thus, Notomys provide an excellent model to evaluate the physiological significance of appetite-regulating hormones in vivo during natural hunger. Many pancreatic and gastrointestinal hormones are implicated in appetite regulation, of which most increase satiety and decrease food intake as exemplified by insulin, cholecystokinin, pancreatic polypeptide, glucagon-like peptide 1 and others [14]. The only exception is ghrelin, which is now known to be a major orexigenic hormone secreted from the stomach [26,27]. The diversity of the anorectic hormones relative to a single orexigenic hormone may reflect the evolutionary history of mammals to combat against starvation as exemplified by the glucose-regulating hormones (insulin versus several hyperglycaemic hormones). Among anorectic hormones, leptin secreted from the white adipose tissue appears to play a central role in suppressing appetite and obesity [28]. Leptin is a product of the obese gene, the disruption of which results in an obese phenotype in mice. Appetite stimulation by increased ghrelin and decreased leptin was observed in Notomys but not in Mus. Comparing the two hormones, the change of leptin more precisely mirrors the actual changes in food intake than that of ghrelin, suggesting the dominance of inhibitory stimulus for appetite regulation.

The current study also demonstrated a coordinated interaction of peripheral and central systems for appetite arousal in vivo in Notomys. Following secretion into the circulation, leptin and ghrelin act on the hypothalamic ARC neurons that are located in a region outside the blood-brain barrier and regulate orexigenic NPY neurons and anorectic α-MSH (POMC) neurons therein [27,28]. The ARC neurons then send their axons to the LH to regulate orexigenic ORX [29] and MCH [30] neurons, and to the PVN where they regulate anorectic CRH, TRH or OXY neurons [12]. We found that in Notomys, the major hypothalamic neuropeptides are regulated in
Figure 3. Changes in the expression of neuropeptide genes in the hypothalamus after WD in (a–g) desert mice (Notomys, n = 6–8) and (h–n) laboratory mice of Swiss strain (Mus, n = 10). The data of Balb-C mice are shown in the electronic supplementary material, figure S2. Data are shown as means ± s.e.m. *p < 0.05 compared with the data before WD (day 0). For abbreviation of neuropeptides, see text.
the direction of appetite stimulation during WD, except for TRH and OXY. Thus, the intimate cooperation of the peripheral and central appetite-regulating systems was demonstrated in the natural hunger exhibited by Notomys. This study also showed that NPY, α-MSH, ORX, MCH and CRH are physiological regulators of appetite in Notomys. It is noteworthy that downregulation of the anorectic POMC and CRH genes more precisely paralleled the change in food intake during WD, as observed for anorectic leptin in plasma; this again suggests the dominance of the anorectic system in appetite regulation in mammals. Consistent with the sustained food intake without drinking water in Mus, the NPY, POMC, MCH and CRH genes changed expression to enhance appetite, but the anorectic TRH and OXY genes are upregulated after WD. As ghrelin and leptin in plasma did not change after WD in Mus, some other factor(s) may regulate the activity of these genes in this species.

There is a report on the appetite regulation in response to food restriction in a desert rodent, the golden spiny mouse, Acomys russatus [31]. Acomys do not store food, unlike Notomys, and thus respond quickly to food restriction. Plasma leptin concentration and energy expenditure decreased after 24 h food restriction with simultaneous increases in the expression of the AgRP and NPY genes. However, the expression of the POMC and CART genes did not change during 17 days of food restriction in Acomys, which differs from profound downregulation of the POMC gene during 12 days of WD when enhanced appetite was evident in Notomys. Leptin administration to the food-restricted Acomys did not affect food intake, but leptin suppressed the NPY gene expression to normal and restored reduced metabolic rate. Therefore, leptin directly regulates the NPY gene expression and metabolic rate independently of food intake. These results suggest that appetite is

Figure 4. Changes in liver and muscle glycogen contents, hepatic glycogen synthase gene expression and plasma glucose and corticosterone concentrations after WD in (a–e) desert mice (Notomys, n = 6–8) and (f–j) laboratory mice of Swiss strain (Mus, n = 10). Data are shown as means ± s.e.m. *p < 0.05 compared with the data before WD (day 0).

regulated not only by the known hormones and neuropeptides, but also by other factors.

We also showed that *Notomys* accumulate a large amount of adipose tissue in the abdomen when water is available, but they consume stored fat rapidly after the onset of WD and start to accumulate glycogen in the liver during prolonged WD. As millet seeds contain 73 per cent carbohydrates as nutrients, water-replete *Notomys* metabolize carbohydrates into lipids for fat storage, but they change the strategy during prolonged WD and use absorbed sugars directly for glycogen deposition for future use. The switching of metabolic substrate from lipid to carbohydrate after long exposure to desiccation is an important strategy for xeric adaptation, because carbohydrate generates more water per oxygen molecule and therefore switching to carbohydrate metabolism could alleviate water loss by respiration in dry environments. The increase in glycogen deposition in *Notomys* is accompanied by a parallel increase in glycogen synthase 2 gene expression and a gradual increase in plasma glucocorticoid (corticosterone) concentration. As corticosterone stimulates both glycolysis and glycogenesis in rodents [32], it could serve as a trigger for switching to carbohydrate metabolism. Such dynamic changes in carbohydrate metabolism were not observed in *Mus*, they continued to accumulate fat and consume hepatic glycogen throughout the 12 day WD period, and plasma corticosterone concentration remained low during WD. Therefore, switching of substrates for oxidation in response to water deficiency appears to be a unique feature of *Notomys* for xeric adaptation. It is intriguing to examine whether such an adaptive strategy for oxidative water production is common to other desert rodents.

An interesting switching of metabolic rate has been reported in the golden spiny mouse in relation to food restriction [33]. The mouse downregulated its resting metabolism and survived on limited food, and then increased metabolism again when sufficient food was provided. A similar response was not observed in the laboratory mouse (*Mus*), as they increased metabolic rate during food restriction. Thus, it was suggested that this ‘metabolic switch’ stimulated by food scarcity is important for survival in the desert, where food availability is limited. In fact, *Notomys* increased body mass in response to supplementary feeding in the wild, suggesting that food availability is limited in nature [34]. In contrast to the food restriction experiments described above, the current study showed that water restriction stimulated food intake, which may lead to an increased metabolic rate for water production. The prolonged WD also changed metabolic substrate utilization from lipids to carbohydrates, presumably for more efficient metabolic water production. Such unique mechanisms for metabolic regulation in response to food and water restriction may enable small rodents to survive in the desert.

We present a schematic summary of the findings of the present study in figure 5. In addition to excellent mechanisms to reduce water loss, we propose that *Notomys* possesses unique mechanisms to increase the gain of water when they are exposed to prolonged water deficiency. At least two mechanisms exist for water production; the mechanism to induce appetite to increase the substrate for oxidation, and the mechanism to switch the substrate for oxidation from fat to carbohydrate. The regulation can be divided into two phases with respect to time: a rapid regulation that occurs soon after WD commences, and a delayed regulation that occurs approximately after 5 days. These abilities of *Notomys* to effectively increase water production may be a characteristic of desert rodents as they were not observed in *Mus*, and they could be a critical adaptation to desert life in addition to the physiological mechanisms to minimize water loss. Furthermore, *Notomys* could serve as an excellent natural animal model for research on obesity and metabolic syndromes.
as they changed appetite and metabolic strategy so drastically in response to WD. We expect that elucidation of the physiological mechanisms for such regulation in Notomys can provide a new insight into the study of energy metabolism in mammals and further contribute to clinical studies on obesity and metabolic syndrome in humans in the future.

All the experiments reported herein were approved by the Deakin University Animal Welfare Committee.

We thank Dr Tes Toop and the members of J.A.D.’s laboratory at Deakin University for their help during Y.T.’s visit, and Adrian Cooper and Briana Spolding from the Metabolic Research Unit at Deakin University for help in measuring body fat. We are also grateful to Dr Takehiro Tsukada of Atmosphere and Ocean Research Institute for the measurement of leptin, Dr Hirohiko Kawai of the National Cardiovascular Center Research Institute for measurement of ghrelin, and Dr Sofie Trajanovska of Deakin University for cDNA cloning of Notomys ghrelin and leptin. Support from Dr Susumu Hyodo and Ms Nanako Hasegawa and an artistic work by Mr Keigo Kakumura of Atmosphere and Ocean Research Institute are also acknowledged. This work was supported by the Japan–Australia Cooperative Programme and by grant-in-aid for Scientific Research (19207005) from the Japan Society for the Promotion of Science to Y.T.

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