

17 **Abstract**

18 In birds little is known about the hormonal signals that communicate nutritional state
19 to the brain and regulate appetitive behaviours. In mammals, the peptide hormones
20 ghrelin and leptin elevate and inhibit consumption and food hoarding, respectively. But
21 in birds, administration of both ghrelin and leptin inhibit food consumption. The role
22 of these hormones in the regulation of food hoarding in avian species has not been
23 examined. To investigate this, we injected wild caught coal tits (*Periparus ater*) with
24 leptin, high-dose ghrelin, low-dose ghrelin and a saline control in the laboratory. We
25 then measured food hoarding and mass gain, as a proxy of food consumption, every 20
26 mins for two hours post-injection. Both high-dose ghrelin and leptin injections
27 significantly reduced hoarding and mass gain compared with controls. Our results
28 provide the first evidence that hoarding behaviour can be reduced by both leptin and
29 ghrelin in a wild bird. These findings add to evidence that the hormonal control of food
30 consumption and hoarding in avian species differs from that in mammals. Food
31 hoarding and consumptive behaviours consistently show the same response to
32 peripheral signals of nutritional state, suggesting that the hormonal regulation of food
33 hoarding has evolved from the consumption regulatory system.

34

35 **Keywords:** caching; energy management; food intake; hormones.

36 **Introduction**

37 Many animals experience fluctuating environments, where food availability varies
38 seasonally. To compensate for this, some species have evolved to store food while
39 availability is high, for later consumption when food is scarce [1]. This behaviour is
40 called food hoarding or caching and is present in multiple taxa [2,3]. Unlike the neural
41 mechanisms that enable animals to retrieve food stores [4], comparatively little is
42 known about the neural and hormonal mechanisms that underpin the motivation to
43 hoard food [5]. In mammals, the peptide hormones ghrelin and leptin control energy
44 balance through stimulating and inhibiting food intake, respectively [5,6]. Food
45 hoarding is likewise regulated in mammals, with ghrelin promoting and leptin
46 suppressing food hoarding [5]. In birds, the hormonal signals that communicate
47 nutritional state to the brain and regulate appetitive behaviours are relatively
48 unexplored. In contrast to mammals, exogenous ghrelin and leptin both inhibit
49 consumption in birds [7,8], suggesting that the hormonal regulation of consumption
50 differs between mammals and birds. The role of these hormones in the regulation of
51 food hoarding in avian species has not been examined. Developing an understanding of
52 the hormonal regulation of food hoarding in birds would further elucidate the
53 contrasting regulation of appetitive and consummatory behaviours between mammals
54 and birds.

55

56 The peptide hormone ghrelin has been identified in a range of avian species, where it
57 is secreted mainly from the proventriculus, but also several other organs within the
58 digestive tract [8,9]. While administration of ghrelin in mammals has a stimulatory
59 effect upon both consumption and food hoarding [5,10–13], in birds central and
60 peripheral administration of ghrelin predominately inhibits food intake [14–17].

61 However, peripheral injection with low-dose ghrelin has also been shown to increase
62 food intake in Japanese quail (*Coturnix japonica*) [17], and food deprivation causes an
63 increase in circulating ghrelin concentrations in both mammals and birds [11,18].
64 Therefore, for the most part ghrelin exerts inhibitory effects upon consumption in birds,
65 but the relationship between ghrelin, energy management and food intake remains
66 equivocal (for review see [7]). Whether ghrelin also exerts inhibitory effects upon food
67 hoarding in birds is still to be investigated.

68

69 In mammals, the peptide hormone leptin plays a significant role in the regulation of
70 food intake and energy balance [6]. Leptin is predominately released from adipose
71 tissue in mammals, where it functions as a signal between peripheral lipid stores and
72 the central nervous system [19]. Both endogenous and exogenous leptin inhibit food
73 intake, and circulating leptin concentrations are positively correlated with an
74 individual's body fat index [5–7]. Leptin also regulates food hoarding motivation in
75 mammals, where peripheral leptin treatment decreases food hoarding in the Siberian
76 hamster, (*Phodopus sungorus*) [20,21]. Similarly in birds, administration of
77 mammalian and 'chicken' leptin inhibits food intake ([22–25] but see [26]). Although
78 a functional leptin receptor has been unequivocally identified in birds [27,28], 'chicken'
79 leptin was incorrectly claimed to have been cloned [29,30]. In fact, the putative
80 'chicken' leptin protein shares 97% amino acid sequence identity to mammalian leptin
81 [31,32]. This lead to controversy surrounding the existence of leptin genes in avian
82 species, and cast doubt on the validity of the behavioural responses to leptin
83 administration seen in birds [7]. However, leptin genes have now been identified in a
84 number of avian species [33–37], and synteny analysis has shown that avian leptin is
85 orthologous to mammalian leptin, despite avian leptin showing limited amino acid

86 sequence identity (~30%) to human and mouse leptin [36]. These findings have
87 resolved the long-standing uncertainty surrounding the existence of leptin genes in
88 birds [7,35], and suggest that leptin may be a candidate hormone for the regulation
89 appetitive behaviours in birds.

90

91 The objective of this study was to evaluate the effect of peripheral injection of leptin,
92 high-dose and low-dose ghrelin upon food hoarding and mass gain in wild caught coal
93 tits (*Periparus ater*). The coal tit is a small woodland passerine, resident from northern
94 Europe to North Africa. Coal tits are a scatter hoarding species, that hoard food
95 throughout the non-breeding season. Based upon the inhibitory effect of ghrelin upon
96 consumption in a number of avian species [8,14], and of leptin in a closely related
97 passerine, the great tit (*Parus major*) [22], we predicted that both leptin and ghrelin
98 would reduce food hoarding and food intake relative to a vehicle control.

99

100 **Methods**

101 *Study subjects and experimental design*

102 In November 2005, free-living coal tits (*Periparus ater*) of unknown sex and age were
103 caught in mist-nets in Northumberland, UK ($n = 9$). Birds were then transported to light
104 and temperature controlled indoor aviaries at Newcastle University, UK. Birds were
105 housed individually in cages (85 x 45 x 95 cm), which were positioned so that birds had
106 visual, but not physical contact. Prior to the experiment, birds were provided daily with
107 *ad-lib* water and a diet of mixed seeds and nuts, wax moths, mealworms, and a scoop
108 of EMP/Universal bird mix. Birds were maintained on winter-like conditions; 8:16 hour
109 light:dark cycle, and at a temperature of 14-19°C, to promote hoarding behaviour
110 [38,39]. To further promote hoarding behaviour, in April 2006 two weeks before the

111 beginning of the experiment, birds were provided with an unpredictable food supply of
112 sunflower seeds, which they had *ad-lib* access to for approximately 40% of their
113 daylight hours, while food was removed for the remaining 60%.

114

115 A repeated measures design was used to investigate whether injection with leptin (10 μ g,
116 630pmol), high-dose ghrelin (ghrelin-H, 0.78 μ g, 250pmol) or low-dose ghrelin
117 (ghrelin-L, 0.16 μ g, 50pmol) affected hoarding behaviour and mass gain relative to
118 control treatment (saline injection). These dosages were based on previous work
119 involving manipulation of ghrelin and leptin in domestic and wild birds [9,17,18, 28,
120 33]. Three birds received an injection on a given day, and each bird had a two-day break
121 between injections. The order of the injections was randomized across birds. Leptin
122 (mouse) was purchased from Protein Laboratories Rehovot, Ltd. (Israel), and ghrelin
123 (chicken, unacylated) was provided by HK, National Cardiovascular Centre Research
124 Institute, Osaka, Japan. Ghrelin and leptin standards were dissolved into 0.05ml of
125 0.75% PBS (pH 7.8). Treatment solutions were made prior to the beginning of the
126 experiment and frozen. They were then thawed immediately before injection.

127

128 Birds were injected into the pectoral muscle just before lights on in the morning. They
129 were then placed back in their individual cages and provided with 50 sunflower seeds
130 and 20 peanut halves within 5 minutes. To provide hoarding sites, wooden blocks with
131 ~100 holes (0.5 x 1cm) were also placed into cages. To measure hoarding activity, the
132 number of hoarded items was counted every 20 minutes for two hours (six-time points).
133 To do this, all food and hoards were removed every 20 minutes. Food was then replaced
134 prior to the next 20 min period. If other areas of the cage in addition to the wooden
135 blocks, such as water bottles or under the cage floor lining, were used to hoard items

136 those were also counted. To measure mass, we used automated balances (Ohaus, Scout
137 Pro 200) attached to the highest perch in each cage. This allowed body mass to be
138 measured automatically each time a bird landed on the perch. The data collected from
139 the electronic balances was noisy as birds moved and landed on the perches. Therefore,
140 the data had to be cleaned prior to analysis. Initially all data outside the range of 6-10
141 g was discarded as unrealistic. Next, we calculated an average based upon the first 5
142 readings per 20 min interval and used this to either include or exclude the next value
143 dependent on whether it deviated by more than 10% in either direction. Then a new
144 average was calculated which included the 5 most recent accepted mass readings. This
145 procedure was used throughout the data set with the moving average allowing for trends
146 throughout the experiment. Both mass and the number of food hoards were recorded
147 for 2 hours post injection.

148

149 *Statistical analyses*

150 To investigate whether hormone treatment influenced hoarding behaviour and mass
151 gain we used Linear Mixed Models (LMMs), with bird ID as a random factor to avoid
152 pseudoreplication. For analysis, we used both accumulative hoarding and mass gain
153 over the two hours, and total hoards and average mass gain per 20 mins. We fitted full
154 models with hormone treatment, time, and treatment*time interaction; *P* values
155 reported are from full models. As hoarding was a count we used a Poisson error
156 structure. Overdispersion was not deemed a problem as the residual mean deviance
157 (residual deviance/residual *d.f.*) was ≤ 1.7 [41]. All analyses were run in R v. 2.12.2,
158 GLMMs and post-hoc Tukey tests were run using the lme4 and multcomp packages
159 [42].

160

161 **Results**

162 Hormone treatment and time post-injection had a significant effect upon accumulative
163 food hoards and mass gain (Figure 1a & 2a, LMM: Hoards: treatment; $\chi^2 = 18.1$, $P <$
164 0.001 , time; $\chi^2 = 109.1$, $P < 0.001$, Mass: treatment; $\chi^2 = 111.3$, $P < 0.001$, time; $\chi^2 =$
165 98.1 , $P < 0.001$). But the interaction between time and treatment was insignificant for
166 both accumulative food hoards and mass gain (Hoards: treatment*time; $\chi^2 = 7.9$, $P =$
167 0.93 , Mass: treatment*time; $\chi^2 = 7.6$, $P = 0.94$). Post-hoc analysis revealed that
168 injection with ghrelin-H and leptin caused coal tits to hoard fewer items, and reduced
169 mass gain compared with the saline control (figure 1a & 2a, table 1). Injection with
170 ghrelin-L did not affect hoarding or mass gain relative to control injection (figure 1a &
171 2a, table 1).

172

173 When we looked at the number of food items hoarded, and mass gain within each 20min
174 period, we found that the effect of hormone treatment differed between the time points,
175 as shown by significant interactions between treatment x time (Hoards; $\chi^2 = 35.4$, $P <$
176 0.01 , Mass; $\chi^2 = 35.4$, $P < 0.01$). To investigate this, we looked at the effect of the
177 hormone treatment within each time point. Injection with ghrelin-H and leptin caused
178 coal tits to hoard fewer items than the saline control at 80-120 min post-injection (figure
179 1b, table 2). Injection with ghrelin-L caused coal tits to hoard more items than the saline
180 control at 60 min post-injection only (figure 1b, table 2). Ghrelin-H and leptin caused
181 coal tits to gain less mass compared with controls at 20-40 min post-injection (figure
182 2b, table 2). Injection with ghrelin-L did not cause mass to differ from controls at any
183 time point.

184

185 **Discussion**

186 To our knowledge, this study is the first to show that both leptin and ghrelin regulate
187 food hoarding and mass gain in a wild bird. As both leptin and ghrelin reduce food
188 hoarding and mass gain, these results further demonstrate that food hoarding and food
189 intake are under different hormonal regulation in birds compared with mammals, where
190 ghrelin elevates and leptin inhibits consumption and food hoarding, respectively [5].
191 While there have been mixed results concerning the effects of ghrelin upon
192 consumption in birds, our results add weight to the majority of studies that show ghrelin
193 has a inhibitory effect upon food intake [8]. Low-dose ghrelin did not affect mass gain
194 relative to controls, which suggests that the dose-dependent effects of peripherally
195 injected ghrelin upon consumption previously reported may not be consistent across
196 avian species [17]. However, low-dose ghrelin did increase hoarding at a single time
197 point relative to controls. Therefore, further investigation of the ghrelin-food hoarding
198 dose-response curve is warranted.

199

200 Importantly, to validate the role of ghrelin in hoarding behaviour, future studies would
201 benefit from measuring circulating ghrelin during high hoarding motivation and
202 validating whether exogenous manipulation results in physiologically relevant
203 increases. However, this is complicated by the fact that ghrelin occurs in two forms,
204 acylated and unacylated (des-acyl: lacking the octanoyl modification at the serine-3).
205 Acylated ghrelin was used in this study, but there are mixed results concerning the
206 influence of acylated and unacylated ghrelin upon consumption in birds. Central and
207 peripheral administration of acylated ghrelin reduces food intake in chickens [8,16]. In
208 layer chicks, central injection of unacylated ghrelin did not affect food intake [43].
209 However, in the garden warbler (*Sylvia borin*) acylated ghrelin did not affect behaviour,
210 while unacylated ghrelin reduced food intake and increased migratory restlessness [14].

211 The biological function of acylated and unacylated ghrelin is also unclear in mammals,
212 where unacylated ghrelin can inhibit or stimulate food intake, and the two molecular
213 forms of ghrelin may regulate appetite through distinct receptors [44–46]. In humans,
214 acylated ghrelin is found at lower concentrations than unacylated ghrelin in the
215 bloodstream [47]. This is because acylated ghrelin binds to the ghrelin receptor in
216 tissues [47], and is rapidly unacylated in the circulation [48,49]. Furthermore, there is
217 currently no method to measure circulating concentrations of unacylated ghrelin in
218 birds [14]. Therefore, how exogenous manipulation of ghrelin influences circulating
219 unacylated ghrelin is unknown, making it difficult to assess the role of acylated and
220 unacylated ghrelin in the regulation of appetitive behaviours in birds.

221

222 The neural regulation of appetite is highly conserved across vertebrate species [50,51].
223 However, how hormonal signals communicate nutritional state to the brain differs
224 between birds and mammals. The peptide neurotransmitter, neuropeptide Y (NPY)
225 mediates the orexigenic effects of ghrelin in rodents [52]. But in birds, hypothalamic
226 NPY expression is unaffected by central injection of ghrelin [53]. Rather in neonatal
227 broiler chicks, central injection of ghrelin elevates plasma corticosterone [53]. This
228 effect was attenuated if astressin, a receptor antagonist for corticotropin-releasing factor
229 (CRF) was co-injected with ghrelin [53]. As CRF suppresses food intake in birds [54],
230 it has been hypothesized that ghrelin may exert its effects upon consumption through
231 CRF, which activates the hypothalamic-pituitary-adrenal (HPA) axis to release
232 corticosterone from the adrenal glands. Therefore, the inhibitory effects of ghrelin upon
233 consumption and food hoarding may be regulated through corticosterone elevation.
234 This is contrary to evidence that elevated corticosterone enhances food hoarding in wild
235 birds [55]. Importantly, CRF is not the only hypothalamic stimulator of the HPA axis,

236 therefore CRF could be acting independently on food intake. Overall, further studies in
237 wild birds that investigate the role of ghrelin and the HPA axis upon hoarding behaviour
238 will be worthwhile.

239

240 Coal tits made fewer hoards and gained less mass when injected with leptin relative to
241 controls. This is consistent with results from hamster species, domesticated and wild
242 birds, where peripheral injection with mammalian leptin causes a reduction in
243 consumption [20–25]. To date, the only hormone that has been shown to influence the
244 motivation to hoard in birds has been corticosterone [55]. Chronic moderate increases
245 of corticosterone increase food consumption, food hoarding and cache retrieval
246 efficiency [1,55]. Both leptin and leptin receptors (LEPR) are expressed in the pituitary
247 and adrenal gland in birds, suggesting that leptin has a role in the stress response and
248 glucocorticoid production [35]. In mammals, the adrenal gland is implicated in the
249 leptin response through the HPA axis [56] and by local secretion of leptin from fat
250 deposits surrounding the adrenal [57]. Therefore, leptin may act through similar circuits
251 in birds and mammals [35].

252

253 In both birds and mammals, expression of NPY and agouti-related protein (AgRP) in
254 neurons within the arcuate nucleus (Arc) of the hypothalamus are elevated in response
255 to food deprivation, and stimulate food intake [7]. Whereas, satiety stimulates
256 expression of pro-opiomelanocortin (POMC) in a different population of Arc neurons.
257 The catabolic effects of POMC are in part caused by stimulating release of α -
258 melanocyte-stimulating hormone (α -MSH) from the anterior pituitary, and its
259 subsequent binding to melanocortin-4 receptors (MC4R) [50]. AgRP has opposing
260 effects to POMC, as it acts as a competitive antagonist with α -MSH on MC4Rs, thereby

261 stimulating food intake [50,51]. In young domestic broiler chickens mammalian leptin
262 does not influence hypothalamic expression of AgRP and POMC, as it does in
263 mammals [58]. Rather, leptin decreased expression of hypothalamic NPY and MC4R,
264 indicating leptin may act directly upon MCRs to regulate food intake in birds [58]. The
265 pattern of leptin and LEPR expression across tissues also differs in birds compared with
266 mammals. Specifically, leptin is not predominately expressed in adipose tissue, as it is
267 in mammals [57]. Also, as mentioned above LEPRs are expressed predominately in the
268 pituitary in birds [34–36]. This is in contrast to mammals, where the hypothalamus is
269 the main site of LEPR expression [35,59]. Furthermore, in a range of bird species leptin
270 has not been detected in the serum, and the expression level of leptin and LEPR show
271 a strong correlation across tissues [28,35,60]. These findings have led to the suggestion
272 that leptin has a more paracrine than endocrine role in birds [7,35,36]. Therefore, it is
273 possible there is an additional mechanism that stimulates local leptin action in the brain
274 and peripheral tissues. Importantly, the systemic administration of leptin used in this
275 study will have triggered LEPRs and shows leptin regulates feeding and hoarding
276 behaviour, but cannot confirm if leptin acts as a hormone in birds. Due to the relatively
277 recent discovery of leptin genes in bird species, further studies are required to
278 investigate how avian leptin peripherally and centrally acts to influence appetitive
279 behaviours in birds [7,36].

280

281 Crucially, the most abundant avian LEPR isoform is orthologous to a mammalian LEPR
282 isoform [7,28], and mammalian leptins activate chicken LEPR cell-line reporter
283 systems [28]. This indicates that the tertiary structure needed to activate the receptor is
284 conserved between mammalian and avian leptin proteins [61]. Therefore, although
285 there is ~30% amino acid sequence identity between mammalian and avian leptins,

286 administration of mammalian leptin likely acts through the avian LEPR, making our
287 behavioural results biologically relevant. However, to validate the role of leptin in
288 appetitive behaviours, future studies would benefit from using recombinant avian leptin
289 for manipulations [7].

290

291 The time frame over which ghrelin and leptin exerted their effects differed between
292 food hoarding and mass gain. Leptin and high-dose ghrelin injection reduced mass gain
293 relative to controls at 20-40 min post-injection, while their effects upon food hoarding
294 were significant 80-120 min post-injection. Similarly in great tits (*Parus major*),
295 control birds initially feed at a higher rate after a night's fast, whereas leptin injection
296 caused an immediate reduction in food intake at lights-on [22]. The rapid effects of
297 peripheral leptin injection upon food intake have also been observed in domesticated
298 chickens [24]. The temporal lag found between the suppression of mass gain and that
299 of hoarding, could be explained if the regulation of hoarding behaviour is
300 physiologically downstream to that of mass gain [19]. Interestingly, in the food
301 hoarding Siberian hamster, the neural circuits that control food intake are also activated
302 more rapidly than those that influence food hoarding, and different brain areas are
303 activated during consumption compared with hoarding behaviour [62]. The similarity
304 of our results suggests that the neural substrates that control consumption, may also
305 differ to those that regulate food hoarding in birds. But the brain regions that regulate
306 food hoarding in birds are yet to be established. Alternatively, as birds spend
307 proportionally more time feeding relative to hoarding initially after lights-on, perhaps
308 their tendency for immediate mass gain influenced our results [63].

309

310 We have shown that short-term food hoarding is influenced by leptin and ghrelin.
311 However, food hoarding Parids also show a seasonal hoarding peak in autumn, in
312 addition to continued low level hoarding throughout winter [1,3]. Unpredictable food,
313 photoperiod and low temperatures have all been shown to stimulate food hoarding in
314 small birds and mammals [1,64]. In autumn food is relatively abundant, but there is also
315 shortening day length and decreasing temperatures. Both short days and low
316 temperatures can increase energetic requirements, by decreasing foraging time and
317 increasing costs of thermoregulation [64]. Therefore, it is possible that peripheral
318 signals of energetic deficit regulate hoarding year-round. If so, hoarding behaviour is
319 more prevalent in autumn than in winter due to high hoarding motivation combined
320 with relatively high food availability. In winter, energetic deficit and motivation to
321 hoard would be higher, but low food availability and shorter daylength prevent
322 increased food hoarding. Interestingly, leptin could also regulate increased hoarding in
323 response to cold temperatures. Chronically cold-exposed Siberian hamsters fed *ad*
324 *libitum* overeat and have reduced circulating leptin concentrations [64]. However,
325 additional hormonal signals may also regulate hoarding behaviour. For example, short-
326 day increases in food intake and food hoarding in Syrian hamsters (*Mesocricetus*
327 *auratus*) are dependent on an intact pineal gland and thus melatonin, as removal of the
328 pineal gland blocks these behavioural responses [65]. Overall, it is likely that multiple
329 environmental factors interact with physiological signals to stimulate seasonal variation
330 in hoarding behaviour.

331

332 Our study adds to evidence that the hormonal control of consumption and hoarding in
333 avian species differs from that in mammals. There is mounting evidence that food
334 hoarding and consumptive behaviours consistently show the same response to leptin

335 and ghrelin [5,66]. This suggests that the hormonal regulation of hoarding behaviour
336 has evolved from the consumption regulatory system. Therefore, the evolutionary
337 emergence of food-hoarding behaviour provides an opportunity to understand how
338 existing physiological mechanisms can be modified to control a behaviour. Studying a
339 convergent behaviour between mammals and birds will further elucidate the
340 mechanisms that regulate appetite.

341 **Table 1.** Results of Tukey tests investigating effects of injection with ghrelin-high, ghrelin-
 342 low or leptin, compared with saline control, upon accumulative hoarding and mass gain in coal
 343 tits. LMMs control for time post injection. Values in bold denotes significance at $P < 0.001$.

Hormone Treatment	Accumulative hoards				Accumulative mass gain			
	Est.	Std. Error	<i>z</i>	<i>P</i>	Est.	Std. Error	<i>t</i>	<i>P</i>
Ghrelin high	-0.22	0.04	-5.35	<0.001	-0.09	0.02	-4.89	<0.001
Ghrelin low	0.04	0.04	0.96	0.77	0.04	0.02	2.26	0.11
Leptin	-0.34	0.04	-8.06	<0.001	-0.13	0.02	-6.93	<0.001

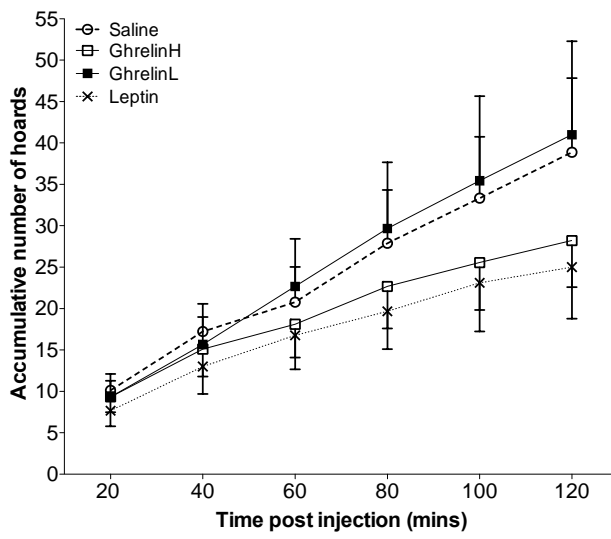
344

345 **Table 2.** Results from LMMs, comparing number of hoards and mean mass in coal tits, after
 346 injection with ghrelin-high, ghrelin-low or leptin, compared with saline control, at six-time
 347 points post-injection (20-120 mins). Values in bold denotes significance at $P < 0.05$, * denotes
 348 marginally non-significant trend $P = 0.05$.

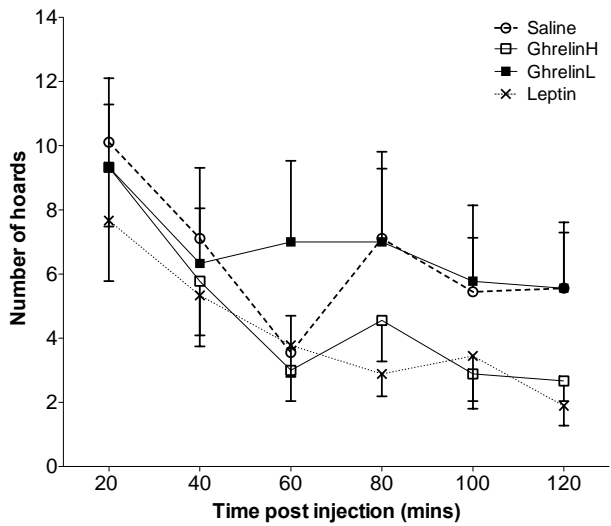
Time	Treatment	Number of hoards				Mean mass			
		Est.	Std. Error	z	P	Est.	Std. Error	t	P
20	Ghrelin high	-0.08	0.15	-0.53	0.60	-0.09	0.03	2.57	0.02
	Ghrelin low	-0.08	0.15	-0.53	0.60	0.02	0.03	0.57	0.58
	Leptin	-0.28	0.16	-1.73	0.08	-0.10	0.03	3.00	0.01
40	Ghrelin high	-0.21	0.19	-1.11	0.27	-0.05	0.02	-2.30	0.03
	Ghrelin low	-0.12	0.18	-0.64	0.53	0.02	0.02	0.89	0.39
	Leptin	-0.29	0.19	-1.51	0.13	-0.07	0.02	-3.21	0.005
60	Ghrelin high	-0.17	0.26	-0.65	0.51	-0.02	0.03	-0.72	0.48
	Ghrelin low	0.68	0.22	3.12	0.002	-0.03	0.03	-1.09	0.29
	Leptin	0.06	0.25	0.25	0.80	-0.06	0.03	-2.14	0.05*
80	Ghrelin high	-0.44	0.20	-2.22	0.03	0.02	0.02	1.27	0.22
	Ghrelin low	-0.02	0.18	-0.09	0.93	0.02	0.02	1.40	0.18
	Leptin	-0.90	0.23	-3.87	<0.001	0.01	0.02	0.54	0.60
100	Ghrelin high	-0.06	0.24	-2.61	0.01	0.01	0.02	0.02	0.98
	Ghrelin low	0.06	0.20	0.30	0.77	0.01	0.02	0.40	0.69
	Leptin	-0.46	0.23	-1.99	0.05*	-0.01	0.02	-0.43	0.67
120	Ghrelin high	-0.73	0.25	-2.95	<0.001	0.02	0.02	1.02	0.32
	Ghrelin low	-0.01	0.20	0.00	0.99	0.02	0.02	1.02	0.32
	Leptin	-1.01	0.28	-5.84	<0.001	0.01	0.02	0.01	0.99

349

A



B



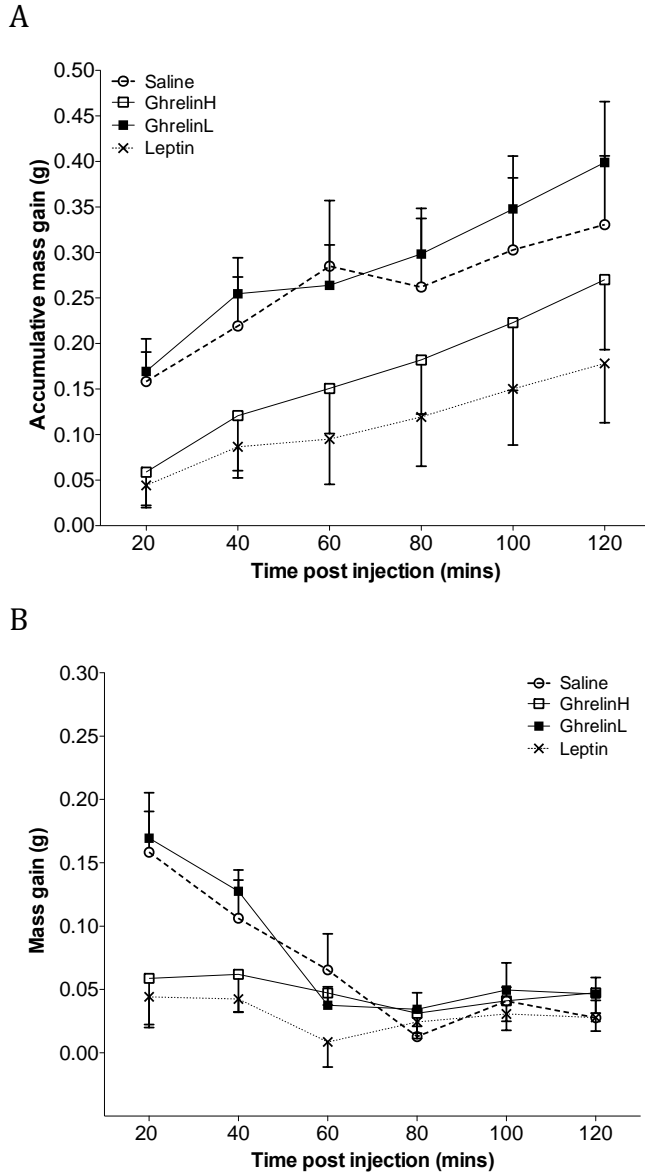
350

351 **Figure 1.** a) The accumulative number of food hoards, and b) the number of food hoards every

352 20 mins, 2 hours after injection with ghrelin-H, ghrelin-L, leptin or saline control in coal tits.

353 Graph displays mean & SE ($n = 9$).

354



355

356 **Figure 2.** a) The accumulative mass gain, and b) mass gain every 20 mins, 2 hours after
 357 injection with ghrelin-H, ghrelin-L, leptin or saline control in coal tits. Graph displays mean &
 358 SE ($n = 9$).

359 **ACKNOWLEDGEMENTS**

360 We thank animal care staff at Newcastle University for their help with animal
361 husbandry and maintenance.

362

363 **AUTHOR CONTRIBUTIONS**

364 LJH analyzed the data, wrote the paper, and prepared figures and tables. RCC and TVS
365 conceived of the experiment, and RCC carried out experiments. HK contributed the
366 avian ghrelin, and TB contributed to the design and conception of the study. All authors
367 reviewed drafts of the paper.

368

369 **DATA ACCESSIBILITY**

370 Data are available to download from Dryad: (doi:10.5061/dryad.ck5p92b).

371

372 **FUNDING**

373 This project was funded by The School of Biology, Newcastle University, UK, and The
374 Engineering and Physical Sciences Research Council (EPSRC), as part of a doctoral
375 thesis by RCC. LJH was funded by a Marie Skłodowska-Curie IF Fellowship.

376

377 **COMPETING INTERESTS**

378 The authors do not have any competing interests.

379

380 **ETHICAL STATEMENT**

381 Animals' care was in accordance with institutional guidelines at the Newcastle
382 University, UK. Birds were captured under an English Nature licence (20042514), and
383 the experiment was performed under a Home Office licence (PPL60/3125).

384 **References**

- 385 1. Pravosudov, V. V & Smulders, T. V 2010 Integrating ecology, psychology and neurobiology
386 within a food-hoarding paradigm. *Philos T Roy Soc B* **365**, 859–867.
- 387 2. Brodin, A. 2010 The history of scatter hoarding studies. *Philos. Trans. R. Soc. London B Biol.*
388 *Sci.* **365**, 869–881.
- 389 3. Vander Wall, S. B. 1990 *Food hoarding in animals*. Chicago, IL: Chicago University Press.
- 390 4. Healy, S. D., de Kort, S. R. & Clayton, N. S. 2005 The hippocampus, spatial memory and food
391 hoarding: a puzzle revisited. *TREE* **20**, 17–22.
392 (doi:<http://dx.doi.org/10.1016/j.tree.2004.10.006>)
- 393 5. Keen-Rhinehart, E., Dailey, M. J. & Bartness, T. 2010 Physiological mechanisms for food-
394 hoarding motivation in animals. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 961–975.
- 395 6. Friedman, J. M. & Halaas, J. L. 1998 Leptin and the regulation of body weight in mammals.
396 *Nature* **395**, 763–770. (doi:10.1038/27376)
- 397 7. Boswell, T. & Dunn, I. C. 2015 Regulation of the avian central melanocortin system and the
398 role of leptin. *Gen. Comp. Endocrinol.* **221**, 278–283. (doi:10.1016/j.ygcen.2014.12.009)
- 399 8. Kaiya, H., Furuse, M., Miyazato, M. & Kangawa, K. 2009 Current knowledge of the roles of
400 ghrelin in regulating food intake and energy balance in birds. *Gen. Comp. Endocrinol.* **163**, 33–
401 38. (doi:10.1016/j.ygcen.2008.11.008)
- 402 9. Kaiya, H., van der Geyten, S., Kojima, M., Hosoda, H., Kitajima, Y., Matsumoto, M.,
403 Geelissen, S., Darras, V. M. & Kangawa, K. 2002 Chicken Ghrelin: Purification, cDNA
404 Cloning, and Biological Activity. *Endocrinology* **143**, 3454–3463. (doi:10.1210/en.2002-
405 220255)
- 406 10. Date, Y., Murakami, N., Toshinai, K., Matsukura, S., Nijima, A., Matsuo, H., Kangawa, K. &
407 Nakazato, M. 2002 The role of the gastric afferent vagal nerve in ghrelin-induced feeding and
408 growth hormone secretion in rats. *Gastroenterology* **123**, 1120–1128.
- 409 11. Higgins, S. C., Gueorguiev, M. & Korbonits, M. 2007 Ghrelin, the peripheral hunger hormone.
410 *Ann. Med.* **39**, 116–136.
- 411 12. Keen-Rhinehart, E. & Bartness, T. J. 2005 Peripheral ghrelin injections stimulate food intake,
412 foraging, and food hoarding in Siberian hamsters. *Am. J. Physiol. - Regul. Integr. Comp.*
413 *Physiol.* **288**, R716–R722.

- 414 13. Wren, A. M. et al. 2001 Ghrelin causes hyperphagia and obesity in rats. *Diabetes* **50**, 2540–
415 2547.
- 416 14. Goymann, W., Lupi, S., Kaiya, H., Cardinale, M. & Fusani, L. 2017 Ghrelin affects stopover
417 decisions and food intake in a long-distance migrant. *Proc. Natl. Acad. Sci.* **114**, 201619565.
418 (doi:10.1073/pnas.1619565114)
- 419 15. Geelissen, S. M., Swennen, Q., Geyten, S. V, Kühn, E. R., Kaiya, H., Kangawa, K., Decuypere,
420 E., Buyse, J. & Darras, V. M. 2006 Peripheral ghrelin reduces food intake and respiratory
421 quotient in chicken. *Domest. Anim. Endocrinol.* **30**, 108–116.
- 422 16. Saito, E., Kaiya, H., Takagi, T., Yamasaki, I., Denbow, D. M., Kangawa, K. & Furuse, M.
423 2002 Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal
424 chicks. *Eur. J. Pharmacol.* **453**, 75–79. (doi:10.1016/S0014-2999(02)02393-2)
- 425 17. Shousha, S., Nakahara, K., Kojima, M., Miyazato, M., Hosoda, H., Kangawa, K. & Murakami,
426 N. 2005 Different effects of peripheral and central ghrelin on regulation of food intake in the
427 Japanese quail. *Gen. Comp. Endocrinol.* **141**, 178–183.
- 428 18. Kaiya, H., Saito, E. S., Tachibana, T., Furuse, M. & Kangawa, K. 2007 Changes in ghrelin
429 levels of plasma and proventriculus and ghrelin mRNA of proventriculus in fasted and refed
430 layer chicks. *Domest. Anim. Endocrinol.* **32**, 247–259.
- 431 19. Houseknecht, K. L., Baile, C. A., Matteri, R. L. & Spurlock, M. E. 1998 The biology of leptin:
432 a review. *J. Anim. Sci.* **76**. (doi:/1998.7651405x)
- 433 20. Buckley, C. A. & Schneider, J. E. 2003 Food hoarding is increased by food deprivation and
434 decreased by leptin treatment in Syrian hamsters. *Am. J. Physiol. - Regul. Integr. Comp.*
435 *Physiol.* **285**, 1021–1029.
- 436 21. Keen-Rhinehart, E. & Bartness, T. J. 2008 Leptin inhibits food-deprivation-induced increases
437 in food intake and food hoarding. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **295**, R1737–
438 R1746.
- 439 22. Löhmus, M., Fredrik Sundström, L., El Halawani, M. & Silverin, B. 2003 Leptin depresses
440 food intake in great tits (*Parus major*). *Gen. Comp. Endocrinol.* **131**, 57–61.
441 (doi:10.1016/s0016-6480(02)00643-3)
- 442 23. Löhmus, M. & Sundström, L. F. 2004 Leptin and social environment influence the risk-taking
443 and feeding behaviour of Asian blue quail. *Anim. Behav.* **68**, 607–612.

- 444 (doi:10.1016/j.anbehav.2003.12.019)
- 445 24. Denbow, D. M., Meade, S., Robertson, A., McMurtry, J. P., Richards, M. & Ashwell, C. 2000
446 Leptin-induced decrease in food intake in chickens. *Physiol. Behav.* **69**, 359–362.
447 (doi:10.1016/S0031-9384(99)00258-9)
- 448 25. Cerasale, D. J., Zajac, D. M. & Guglielmo, C. G. 2011 Behavioral and physiological effects of
449 photoperiod-induced migratory state and leptin on a migratory bird, *Zonotrichia albicollis*: I.
450 Anorectic effects of leptin administration. *Gen. Comp. Endocrinol.* **174**, 276–286.
451 (doi:10.1016/J.YGCEN.2011.08.025)
- 452 26. Bungo, T., Shimojo, M., Masuda, Y., Tachibana, T., Tanaka, S., Sugahara, K. & Furuse, M.
453 1999 Intracerebroventricular administration of mouse leptin does not reduce food intake in the
454 chicken. *Brain Res.* , 196–198.
- 455 27. Adachi, H., Takemoto, Y., Bungo, T. & Ohkubo, T. 2008 Chicken leptin receptor is functional
456 in activating JAK-STAT pathway in vitro. *J. Endocrinol.* **197**, 335–42. (doi:10.1677/JOE-08-
457 0098)
- 458 28. Hen, G., Yosefi, S., Ronin, A., Einat, P., Rosenblum, C. I., Denver, R. J. & Friedman-Einat, M.
459 2008 Monitoring leptin activity using the chicken leptin receptor. *J. Endocrinol.* **197**, 325–333.
460 (doi:10.1677/JOE-08-0065)
- 461 29. Taouis, M., Chen, J.-W., Daviaud, C., Dupont, J., Derouet, M. & Simon, J. 1998 Cloning the
462 chicken leptin gene. *Gene* **208**, 239–242. (doi:10.1016/S0378-1119(97)00670-7)
- 463 30. Ashwell, C. M., Czerwinski, S. M., Brocht, D. M. & McMurtry, J. P. 1999 Hormonal
464 regulation of leptin expression in broiler chickens. *Am. J. Physiol. Integr. Comp. Physiol.* **276**,
465 R226–R232. (doi:10.1152/ajpregu.1999.276.1.R226)
- 466 31. Friedman-Einat, M., Boswell, T., Horev, G., Girishvarma, G., Dunn, I. C., Talbot, R. T. &
467 Sharp, P. J. 1999 The chicken leptin gene: has it been cloned? *Gen. Comp. Endocrinol.* **115**,
468 354–63. (doi:10.1006/gcen.1999.7322)
- 469 32. Pitel, F., Faraut, T., Bruneau, G. & Monget, P. 2010 Is there a leptin gene in the chicken
470 genome? Lessons from phylogenetics, bioinformatics and genomics. *Gen. Comp. Endocrinol.*
471 **167**, 1–5. (doi:10.1016/j.ygcen.2009.10.006)
- 472 33. Prokop, J. W. et al. 2014 Discovery of the elusive leptin in birds: Identification of several
473 ‘missing links’ in the evolution of leptin and its receptor. *PLoS One* **9**, e92751.

- 474 (doi:10.1371/journal.pone.0092751)
- 475 34. Friedman-Einat, M., Cogburn, L. A., Yosefi, S., Hen, G., Shinder, D., Shirak, A. & Seroussi, E.
476 2014 Discovery and characterization of the first genuine avian leptin gene in the rock dove
477 (*Columba livia*). *Endocrinology* **155**, 3376–3384. (doi:10.1210/en.2014-1273)
- 478 35. Seroussi, E., Cinnamon, Y., Yosefi, S., Genin, O., Gage Smith, J., Rafati, N., Bornelöv, S.,
479 Andersson, L. & Friedman-Einat, M. 2015 Identification of the long-sought leptin in chicken
480 and duck: expression pattern of the highly GC-rich avian leptin fits an autocrine/paracrine
481 rather than endocrine function. *Endocrinology* **157**, 737–751. (doi:10.1210/en.2015-1634)
- 482 36. Huang, G., Li, J., Wang, H., Lan, X. & Wang, Y. 2014 Discovery of a novel functional leptin
483 protein (LEP) in zebra finches: Evidence for the existence of an authentic avian leptin gene
484 predominantly expressed in the brain and pituitary. *Endocrinology* **155**, 3385–3396.
485 (doi:10.1210/en.2014-1084)
- 486 37. Friedman-Einat, M. & Seroussi, E. 2014 Quack leptin. *BMC Genomics* **15**, 551.
487 (doi:10.1186/1471-2164-15-551)
- 488 38. Clayton, N. S. & Cristol, D. A. 1996 Effects of photoperiod on memory and food storing in
489 captive marsh tits, *Parus palustris*. *Anim. Behav.* **52**, 715–726.
490 (doi:http://dx.doi.org/10.1006/anbe.1996.0216)
- 491 39. Shettleworth, S. J., Hampton, R. R. & Westwood, R. P. 1995 Effects of season and photoperiod
492 on food storing by black-capped chickadees, *Parus atricapillus*. *Anim. Behav.* **49**, 989–998.
- 493 40. Buyse, J., Janssen, S., Geelissen, S., Swennen, Q., Kaiya, H., Darras, V. M. & Dridi, S. 2009
494 Ghrelin modulates fatty acid synthase and related transcription factor mRNA levels in a tissue-
495 specific manner in neonatal broiler chicks. *Peptides* **30**, 1342–1347.
496 (doi:10.1016/j.peptides.2009.04.015)
- 497 41. Crawley, M. J. 2012 *The R book, 2nd Edition*. Chichester, England: John Wiley & Sons Ltd.
- 498 42. 2012 R: a language and environment for statistical computing.
- 499 43. Tachibana, T., Tanaka, M. & Kaiya, H. 2011 Central injection of des-acyl chicken ghrelin does
500 not affect food intake in chicks. *Gen. Comp. Endocrinol.* **171**, 183–188.
501 (doi:10.1016/j.ygcen.2011.01.008)
- 502 44. Toshinai, K. et al. 2006 Des-acyl ghrelin induces food intake by a mechanism independent of
503 the growth hormone secretagogue receptor. *Endocrinology* **147**, 2306–2314.

- 504 (doi:10.1210/en.2005-1357)
- 505 45. Asakawa, A., Inui, A., Fujimiya, M., Sakamaki, R., Shinfuku, N., Ueta, Y., Meguid, M. M. &
506 Kasuga, M. 2005 Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin.
507 *Gut* **54**, 18–24. (doi:10.1136/gut.2004.038737)
- 508 46. Chen, C.-Y., Inui, A., Asakawa, A., Fujino, K., Kato, I., Chen, C.-C., Ueno, N. & Fujimiya, M.
509 2005 Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in
510 conscious rats. *Gastroenterology* **129**, 8–25. (doi:10.1053/J.GASTRO.2005.04.015)
- 511 47. Hosoda, H., Doi, K., Nagaya, N., Okumura, H., Nakagawa, E., Enomoto, M., Ono, F. &
512 Kangawa, K. 2004 Optimum collection and storage conditions for ghrelin measurements:
513 octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples.
514 *Clin. Chem.* **50**, 1077–80. (doi:10.1373/clinchem.2003.025841)
- 515 48. Satou, M., Nishi, Y., Yoh, J., Hattori, Y. & Sugimoto, H. 2010 Identification and
516 characterization of acyl-protein thioesterase 1/lysophospholipase I as a ghrelin deacylation/
517 lysophospholipid hydrolyzing enzyme in fetal bovine serum and conditioned medium.
518 *Endocrinology* **151**, 4765–4775. (doi:10.1210/en.2010-0412)
- 519 49. Akamizu, T., Shinomiya, T., Irako, T., Fukunaga, M., Nakai, Y., Nakai, Y. & Kangawa, K.
520 2005 Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy
521 subjects using a new direct ELISA assay. *J. Clin. Endocrinol. Metab.* **90**, 6–9.
522 (doi:10.1210/jc.2004-1640)
- 523 50. Boswell, T. & Dunn, I. C. 2017 Regulation of agouti-related protein and pro-opiomelanocortin
524 gene expression in the avian arcuate nucleus. *Front. Endocrinol. (Lausanne)*. **8**, 75.
525 (doi:10.3389/fendo.2017.00075)
- 526 51. Schneider, J. E., Wise, J. D., Benton, N. A., Brozek, J. M. & Keen-Rhinehart, E. 2013 When do
527 we eat? Ingestive behavior, survival, and reproductive success. *Horm. Behav.* **64**, 702–728.
528 (doi:10.1016/j.yhbeh.2013.07.005)
- 529 52. Mondal, M. S., Date, Y., Yamaguchi, H., Toshinai, K., Tsuruta, T., Kangawa, K. & Nakazato,
530 M. 2005 Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. *Regul.*
531 *Pept.* **126**, 55–59. (doi:10.1016/j.regpep.2004.08.038)
- 532 53. Saito, E.-S., Kaiya, H., Tachibana, T., Tomonaga, S., Denbow, D. M., Kangawa, K. & Furuse,
533 M. 2005 Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing

534 factor system in neonatal chicks. *Regul. Pept.* **125**, 201–208.
535 (doi:10.1016/J.REGPEP.2004.09.003)

536 54. Zhang, R., Nakanishi, T., Ohgushi, A., Ando, R., Yoshimatsu, T., Denbow, D. M. & Furuse,
537 M. 2001 Suppression of food intake induced by corticotropin-releasing factor family in
538 neonatal chicks. *Eur. J. Pharmacol.* **427**, 37–41. (doi:10.1016/S0014-2999(01)01109-8)

539 55. Pravosudov, V. V 2003 Long-term moderate elevation of corticosterone facilitates avian food-
540 caching behavior and enhances spatial memory. *Proc. R. Soc. B-Biological Sci.* **270**, 2599–
541 2604.

542 56. Malendowicz, L. K., Rucinski, M., Belloni, A. S., Ziolkowska, A. & Nussdorfer, G. G. 2007
543 Leptin and the regulation of the hypothalamic-pituitary-adrenal axis. In *International Review of*
544 *Cytology*, pp. 63–102. Academic Press. (doi:http://dx.doi.org/10.1016/S0074-7696(07)63002-
545 2)

546 57. Letizia, C. et al. 2014 Leptin and adiponectin mRNA expression from the adipose tissue
547 surrounding the adrenal neoplasia. *J. Clin. Endocrinol. Metab.* **100**, E101–E104.
548 (doi:10.1210/jc.2014-2274)

549 58. Dridi, S., Swennen, Q., Decuypere, E. & Buyse, J. 2005 Mode of leptin action in chicken
550 hypothalamus. *Brain Res.* **1047**, 214–223. (doi:10.1016/J.BRAINRES.2005.04.034)

551 59. Scott, M. M., Lachey, J. L., Sternson, S. M., Lee, C. E., Elias, C. F., Friedman, J. M. &
552 Elmquist, J. K. 2009 Leptin targets in the mouse brain. *J. Comp. Neurol.* **514**, 518–532.
553 (doi:10.1002/cne.22025)

554 60. Yosefi, S., Hen, G., Rosenblum, C. I., Cerasale, D. J., Beaulieu, M., Criscuolo, F. & Friedman-
555 Einat, M. 2010 Lack of leptin activity in blood samples of Adélie penguin and bar-tailed
556 godwit. *J. Endocrinol.* **207**, 113–22. (doi:10.1677/JOE-10-0177)

557 61. Prokop, J. W., Duff, R. J., Ball, H. C., Copeland, D. L. & Londraville, R. L. 2012 Leptin and
558 leptin receptor: Analysis of a structure to function relationship in interaction and evolution
559 from humans to fish. *Peptides* **38**, 326–336. (doi:10.1016/j.peptides.2012.10.002)

560 62. Teubner, B. J. W., Keen-Rhinehart, E. & Bartness, T. J. 2012 Third ventricular coinjection of
561 subthreshold doses of NPY and AgRP stimulate food hoarding and intake and neural
562 activation. *AJP Regul. Integr. Comp. Physiol.* **302**, R37–R48.
563 (doi:10.1152/ajpregu.00475.2011)

- 564 63. Lucas, J. R., Peterson, L. J. & Boudinier, R. L. 1993 The effects of time constraints and
565 changes in body mass and satiation on the simultaneous expression of caching and diet-choice
566 decisions. *Anim. Behav.* **45**, 639–658. (doi:http://dx.doi.org/10.1006/anbe.1993.1080)
- 567 64. Bartness, T. J., Keen-Rhinehart, E., Dailey, M. J. & Teubner, B. J. 2011 Neural and hormonal
568 control of food hoarding. *Am J Physiol Regul Integr Comp Physiol* **301**, 641–655.
569 (doi:10.1152/ajpregu.00137.2011.—Many)
- 570 65. Fleming, A. S., Scardicchio, D. S. & Scardicchio, L. G. 1986 Photoperiodic and pineal effects
571 on food intake, food retrieval, and body weight in female Syrian hamsters. *J. Biol. Rhythms* **1**,
572 285–301. (doi:10.1177/074873048600100403)
- 573 66. Keen-Rhinehart, E., Ondek, K. & Schneider, J. E. 2013 Neuroendocrine regulation of
574 appetitive ingestive behavior. *Front. Neurosci.* (doi:10.3389/fnins.2013.00213)
- 575