Ghrelin affects stopover decisions and food intake in a long-distance migrant

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Billions of birds migrate long distances to either reach breeding areas or to spend the winter at more benign places. On migration, most passerines frequently stop over to rest and replenish their fuel reserves. To date, we know little regarding how they decide that they are ready to continue their journey. What physiological signals tell a bird's brain that its fuel reserves are sufficient to resume migration? A network of hormones regulates food intake and body mass in vertebrates, including the recently discovered peptide hormone, ghrelin. Here, we show that ghrelin reflects body condition and influences migratory behavior of wild birds. We measured ghrelin levels of wild garden warblers (*Sylvia borin*) captured at a stopover site. Further, we manipulated blood concentrations of ghrelin to test its effects on food intake and migratory restlessness. We found that acylated ghrelin concentrations of garden warblers with larger fat scores were higher than those of birds without fat stores. Further, injections of unacylated ghrelin decreased food intake and increased migratory restlessness. These results represent experimental evidence that appetite-regulating hormones control migratory behavior. Our study lays a milestone in migration physiology because it provides the missing link between ecologically dependent factors such as condition and timing of migration. In addition, it offers insights in the regulation of the hormonal system controlling food intake and energy stores in vertebrates, whose disruption causes eating disorders and obesity.

bird migration | ghrelin | migratory restlessness | food intake | stopover

Every year, billions of birds migrate long distances to either reach areas offering favorable wintering conditions or breeding grounds that thrive with food during the reproductive season. For most small passerines, migration involves frequent stopovers to rest and refuel. During stopover, birds are exposed to fluctuating environmental conditions, including weather and food, which influence the duration of their stay. However, the final decision to resume migration depends on the interplay between environmental conditions, the endogenous program, and the physiological condition of an individual. Theory predicts that stopover decisions depend on amount and rate of deposition of energy stores (1, 2), and empirical findings have confirmed this hypothesis (3–12). The big and yet unanswered question is, How do birds know that they are ready to depart? That is, which physiological signals tell the brain that the fuel reserves are sufficient to resume migration? This link that connects physiological condition and brain is still unknown.

Previous work has shown that fat stores are the best predictor for migratory disposition and stopover duration: Birds with larger subcutaneous (s.c.) fat stores show more migratory restlessness in captivity (8, 10, 13, 14), stay shorter times at stopover sites (9, 11), and migrate faster (12). Because birds fuel their migratory flights primarily through fatty acid metabolism (15–17), we expect that factors reflecting fuel load or the energetic condition must be involved in the behavioral switch between resting at stopover sites and resuming migration.

Complex networks of hormones regulate food intake and appetite in vertebrates (18–20). The hormones leptin, obestatin, adiponectin, orexin, and ghrelin have been shown to regulate behavioral and physiological parameters such as food intake, lipid storage, and lipid metabolism in mammals (e.g., refs. 21, 22). In birds, much less is known about the function of these hormones. Recently, avian ghrelin has been identified and its role described in domestic avian species (23–26). In birds, this peptide hormone is secreted from the proventriculus (the glandular part of the stomach) and other organs of the digestive tract, for example, pancreas, duodenum, spleen, and liver. Hence, ghrelin is one of the molecules that communicate the nutritional state of the organism to control centers in the brain. Previous studies in mammals and domestic poultry have shown that ghrelin affects appetite, food intake, lipid metabolism, and energy expenditure (27).

Although the structure of ghrelin is widely conserved among vertebrates, its main functions differ among taxa. For example, in rodents ghrelin promotes conservation of energy in lipid stores (28, 29) by enhancing the utilization of carbohydrates and reducing the utilization of fat, demonstrated by an increase of fat mass and respiratory quotient after s.c. ghrelin injections (29). In contrast, ghrelin reduced the respiratory quotient in broiler chicken (30), suggesting that ghrelin reduces the build-up of fat stores in birds. This finding was confirmed by another study showing that in broiler chicken peripheral administration of ghrelin down-regulates mRNA levels of the lipogenic enzyme fatty acid synthase (FAS) and its transcription factors in the liver, the major site for building up fat stores in birds (31). Further, ghrelin

Significance

Twice a year, billions of birds migrate across continents. Along their route, most species spend considerable time at stopover sites to replenish their fuel stores. What physiological signals tell them when they are ready to continue their journey? Ghrelin is a recently discovered hormone involved in appetite regulation. We found that ghrelin concentrations correlated positively with fat stores of wild garden warblers. Further, birds injected with ghrelin decreased their food intake and increased their drive to continue migration. Hence, our study shows that hormones regulating food intake and energy stores control migratory behavior. This is a previously unknown role for a hormonal system shared by birds and mammals, whose disruption causes eating disorders and obesity.

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stimulates food intake in mammals (32–34), whereas it inhibits food intake in quail and chicken (24, 35–38).

Here, we studied the role of ghrelin in regulating migratory behavior of wild birds. Specifically, we investigated whether ghrelin reflects the nutritional state (i.e., fat stores) and examined if it regulates the expression of migratory behavior in a long-distance migrant, the garden warbler (*Sylvia borin*). A robust prediction of the relationship between circulating ghrelin and fat stores was difficult to formulate, because little is known about this relationship so far. Haqq et al. (39) found that in humans ghrelin negatively correlates with the body mass index (BMI), whereas another study showed no correlation between ghrelin and BMI, fat mass, fat-free mass, and percentage body fat (40). Nevertheless, based on previous studies in quail and chicken, we predicted that ghrelin communicates the nutritional state to the brain by inhibiting food intake and by increasing the amount of migratory restlessness (or *Zugunruhe*), defined as the urge of captive birds to migrate (e.g., ref. 41). We measured circulating concentrations of ghrelin in free-living garden warblers captured at a stopover site and analyzed ghrelin concentrations in relation to s.c. fat stores. Next, we experimentally manipulated ghrelin concentrations in garden warblers temporarily housed in cages and studied the effect of these manipulations on food intake and amount of *Zugunruhe*.

Results

Ghrelin and Fat Reserves. Birds with a fat score of 1, 2, or 3 had higher levels of acylated ghrelin than birds with no visible fat stores—that is, fat score 0 (Fig. 1). The latency between capture and blood sampling had a slight positive effect on ghrelin levels [fat score effect, $F_{3,\ 63} = 4.155$; slope of time between capture and blood sampling, 0.054 (0.004; 0.104), $F_{1,\ 63} = 3.228$; overall model adjusted $R^2 = 0.15$].

Unacylated Ghrelin Treatment and *Zugunruhe*. Treatment with both 2 μmol/L and 10 μmol/L of unacylated ghrelin increased the amount of *Zugunruhe* compared with a saline treatment (Fig. 2). Condition influenced *Zugunruhe* in saline-treated birds [Fig. 2*A*; $\log_{10}$ slope, 0.212 (0.091; 0.336)] and in birds that were treated with 2 μmol/L unacylated ghrelin [Fig. 2*B*; $\log_{10}$ slope, 0.177 (0.072; 0.283)]. Condition did not influence *Zugunruhe* in birds that were treated with 10 μmol/L of unacylated ghrelin [Fig. 2*C*; $\log_{10}$ slope, 0.090 (–0.031; 0.209); overall model statistics: treatment effect, $F_{2,\ 113} = 5.532$; condition effect, $F_{1,\ 113} = 23.244$; Treatment × Condition interaction, $F_{2,\ 113} = 1.067$; adjusted $R^2 = 0.21$]. Because particularly birds in low body condition normally showed little *Zugunruhe*, these data suggest that especially high levels of ghrelin were able to override any effect of condition on *Zugunruhe* (Fig. 2). Using fat score instead of condition as a covariate rendered similar results (see Table S1).

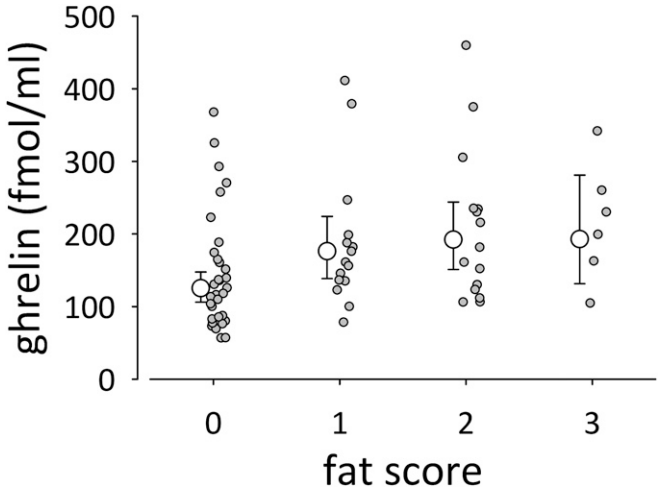

Fig. 1. Ghrelin and fat score of garden warblers. Filled smaller circles indicate individual data points, whereas the larger open circles and the error bars indicate mean estimates and their 95% credible intervals. The 95% credible intervals of the birds with fat scores 1, 2, and 3 did not overlap with the mean estimate of birds with a fat score of 0, indicating that birds with more body fat were more likely to express higher levels of ghrelin.

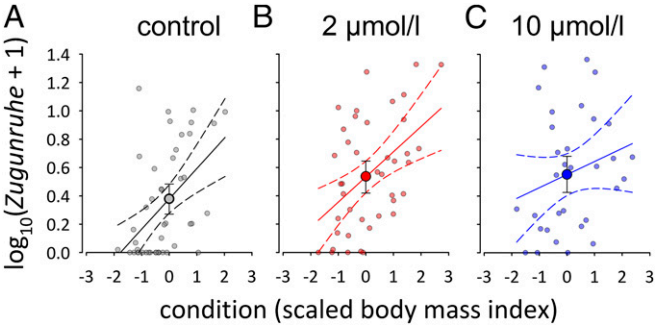

Fig. 2. Effect of saline (*A*) and 2 μmol/L (*B*) and 10 μmol/L (*C*) unacylated ghrelin on *Zugunruhe* in relation to condition. Both doses of unacylated ghrelin increased *Zugunruhe*. In addition, *Zugunruhe* showed a strong condition dependence in saline-treated birds [slope, 0.22 (0.10; 0.34)] and in birds treated with 2 μmol/L unacylated ghrelin [slope, 0.18 (0.07; 0.28)]. In birds treated with 10 μmol/L unacylated ghrelin, the treatment effect overrode any effect of condition [slope, 0.10 (–0.03; 0.21)]. The smaller dots indicate individual data points, whereas the large circles with error bars refer to the respective group means and their 95% credible intervals. The 95% credible intervals of the 2 μmol/L unacylated ghrelin treatment and those of the 10 μmol/L unacylated ghrelin treatment did not overlap with the mean estimate of the saline group, indicating that unacylated ghrelin increased *Zugunruhe* compared with the saline treatment.

Effect of Unacylated Ghrelin on Food Intake. Treatment with unacylated ghrelin affected food intake depending on the dosage. Birds that received 10 μmol/L of unacylated ghrelin had a lower food intake than saline-treated birds (Fig. 3 *A* and *C*) when controlled for condition (effect of food intake, $F_{2,\ 115} = 3.4835$; condition effect, $F_{1,\ 115} = 13.35$; overall model adjusted $R^2 = 0.13$). Unacylated ghrelin mainly affected the food intake of birds in poor condition, which again suggests that unacylated ghrelin overrode any effect of condition, because food intake is already low in birds with good condition and thus any effect of unacylated ghrelin would be expected to mainly affect birds in poor condition (Fig. 3). Replacing condition with fat score as a covariate rendered even stronger results (see Table S2).

Effects of Acylated Ghrelin Treatment on *Zugunruhe* and Food Intake. Condition had a strong effect on *Zugunruhe* in saline-treated (Fig. 4*A*) but not in birds treated with either 2 μmol/L or 10 μmol/L ghrelin (Fig. 4 *B* and *C*). When controlled for condition, treatment with 2 μmol/L acylated ghrelin did not affect the amount of *Zugunruhe* compared with saline treatment (Fig. 4 *A* and *B*), but 10 μmol/L of acylated ghrelin reduced the amount of *Zugunruhe* compared with the saline treatment (Fig. 4*C*). The absence of condition dependence suggests that acylated ghrelin mainly affected birds in good body condition, because birds in poor condition showed little *Zugunruhe* to begin with. However, the model explained only a small proportion of the variance in the data, suggesting a rather weak effect of acylated ghrelin on *Zugunruhe* (treatment effect, $F_{2,\ 111} = 2.196$; condition effect, $F_{1,\ 111} = 4.129$; interaction, $F_{2,\ 111} = 0.745$; overall model, $F_{5,\ 111} = 2.002$; adjusted $R^2 = 0.04$). Using fat score instead of condition as a covariate rendered similar results (see Table S3).

Treatment with either dose of acylated ghrelin did not affect food intake when controlled for condition (treatment effect, $F_{2,\ 111} = 0.780$;

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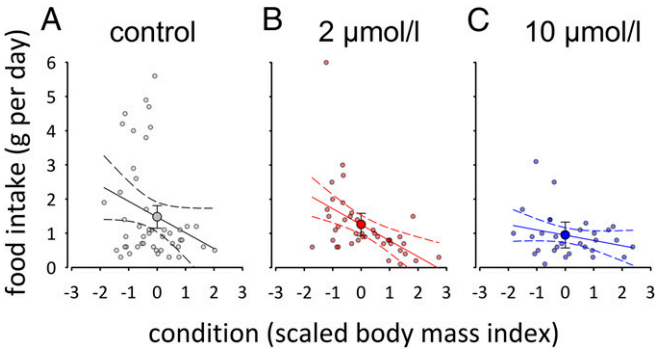

Fig. 3. Effect of saline (*A*) and 2 µmol/L (*B*) and 10 µmol/L (*C*) unacylated ghrelin on food intake in relation to condition. Food intake showed a strong condition dependence in saline-treated birds [slope, –0.46 (–0.84; –0.09)] and in birds treated with 2 µmol/L unacylated ghrelin [slope, –0.47 (–0.79; –0.14)] but not in birds treated with 10 µmol/L unacylated ghrelin [slope, –0.15 (–0.51; 0.21)], in which the treatment effect overrode any effect of condition. The smaller dots indicate individual data points, whereas the larger dots with error bars refer to the respective group means and their 95% credible intervals. The 95% credible intervals of the 10 µmol/L unacylated ghrelin treatment did not overlap with the mean estimate of the control group, indicating that unacylated ghrelin decreased food intake.

condition effect, $F_{1,\ 111} = 1.289$; interaction, $F_{2,\ 111} = 0.369$; overall model, $F_{5,\ 111} = 0.717$; adjusted $R^2 = 0.01$; see Table S4). Using fat score instead of condition as a covariate also showed that treatment did not have an impact on food intake but showed that fatter birds ate less than leaner birds (see Table S5).

Discussion

This study showed that circulating acylated ghrelin reflects s.c. fat stores in wild migrating garden warblers caught at a stopover site. Further, administration of unacylated and acylated ghrelin influenced food intake and the amount of migratory restlessness (*Zugunruhe*). In particular, unacylated ghrelin increased *Zugunruhe* and reduced food intake specifically in animals with a lower body condition that typically eat more than birds in good body condition. Acylated ghrelin had no effect on food intake but decreased *Zugunruhe* of birds in good body condition, particularly when administered at the higher dose. This study measured ghrelin concentrations in wild birds and provided evidence for a role of ghrelin and of appetite-stimulating or -suppressing (orexic and anorexic) hormones in general in the regulation of migratory behavior and decision-making at stopover sites.

The association between circulating ghrelin concentrations and visible body fat indicates ghrelin as a predictor of the extent of body fat stores. This finding confirmed our hypothesis about high ghrelin levels in fat individuals, suggested by previous studies that showed reduced build-up of fat stores in ghrelin-administered domestic birds (30, 31). Increased ghrelin will activate the breakdown of lipids in the liver, which is necessary to resume migration because fat is the primary fuel for migrating birds and induces migratory behavior (*Zugunruhe*). This interpretation fits very well with a number of studies that demonstrated that fat stores are among the best physiological predictors of stopover duration (3–12).

Contrary to our expectations, peripheral injections of acylated ghrelin did not influence feeding behavior and only weakly affected *Zugunruhe* (i.e., by mainly decreasing *Zugunruhe* of birds in good condition). This finding does not support the hypothesis that acylated ghrelin suppresses food intake in migratory passerines. Previous studies on domestic poultry had shown that ghrelin administration induces a short-term reduction in food intake, both when given peripherally into the body cavity or centrally into the cerebral ventricle (23, 30, 31, 35, 36, 42, 43). However, low doses had the opposite effect in Japanese quail (38), suggesting a more complicated relationship between the hormone and behavior.

In contrast to acylated ghrelin, unacylated ghrelin effectively increased *Zugunruhe* and inhibited food intake in garden warblers. Currently, the biological functions of unacylated ghrelin are largely unknown, and at present there is no method to determine the levels of this hormone in birds. The radioimmunoassay (RIA) we used in the present study is known as the *N*-RIA (44), and the antibody used in this RIA is able to recognize the portion 1–11 of rat ghrelin including the acyl modification at serine 3, which is conserved in vertebrates. Therefore, unacylated ghrelin cannot be measured with this assay. The C-terminal is less conserved, and assays developed for mammalian unacylated ghrelin based on antibodies that bind to the C-terminal do not recognize avian ghrelin. In humans, the concentrations of acylated and unacylated ghrelin are strongly correlated (45), but in general, little is known regarding the relationship between acylated and unacylated ghrelin (46). Acylated ghrelin rapidly disappears from the circulation because it binds to the ghrelin receptor in systemic tissues (45) and because it is rapidly unacylated in the circulation (47, 48). In mammals, the majority (80–90%) of the hormone secreted into the blood is unacylated ghrelin (49–51). Until recently, unacylated ghrelin was considered to be an inactive form of ghrelin, but new evidence indicates that it might be biologically active and may be involved in the regulation of appetite through a receptor distinct from the ghrelin receptor (52–54). In mammals, unacylated ghrelin can either inhibit (52, 53) or stimulate (54) food intake. The only study conducted in birds showed no effects of central administration of unacylated ghrelin on feeding behavior in neonatal chicken (55).

Several observations, however, suggest a mechanism of action for unacylated ghrelin in the brain. In mice, circulating unacylated ghrelin easily passes the blood–brain barrier and accumulates in the brain (56). The brain of adult chickens shows substantial levels of expression of ghrelin-O-acetyltransferase (GOAT), the enzyme responsible for the conversion of unacylated ghrelin into acylated ghrelin (57). GOAT expression interestingly is present in areas that express also the receptor for acylated ghrelin (growth hormone secretagogue receptor 1a, or GHSR1a)—that is, the hypothalamus (57), which is the main region regulating food intake in mammals and birds (36, 58). Thus, it is possible that unacylated ghrelin enters the brain and is locally acylated to act on centers

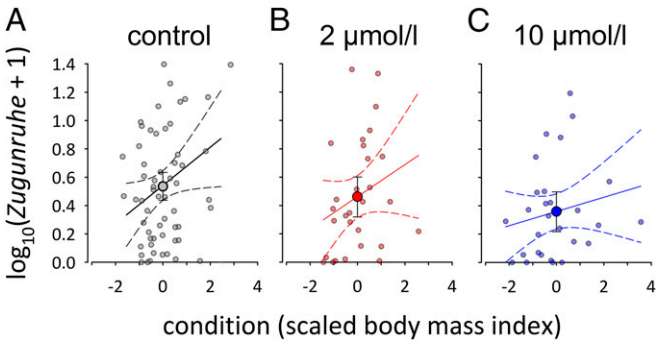

Fig. 4. Effect of saline (*A*) and 2 µmol/L (*B*) and 10 µmol/L (*C*) acylated ghrelin on *Zugunruhe* in relation to condition. *Zugunruhe* showed a strong condition dependence in saline-treated birds [slope, 0.11 (0.01; 0.23)], but this condition dependence disappeared in birds treated with acylated ghrelin [slope 2 µmol/L, 0.11 (–0.04; 0.27); slope 10 µmol/L, 0.05 (–0.07; 0.17)], suggesting that treatment with acylated ghrelin mainly affected birds in good body condition. The smaller dots indicate individual data points, whereas large dots with error bars refer to the respective group means and their 95% credible intervals. The 95% credible intervals of the 10 µmol/L acylated ghrelin treatment did not overlap with the mean estimate of the saline group, indicating that 10 µmol/L acylated ghrelin reduced *Zugunruhe* compared with the saline treatment. The 2 µmol/L treatment ranged in between.

regulating food intake. Alternatively, unacylated ghrelin may exert its action via a so far unknown pathway, explaining the opposing effects of acylated and unacylated ghrelin on *Zugunruhe*.

In the present study, unacylated ghrelin reduced food intake in garden warblers, but the effects were stronger in birds with a low scaled BMI—that is, birds in poor condition that typically eat more. Unacylated ghrelin had little effect on birds in good body condition that typically eat little to start with (exemplified by the control birds in Fig. 3). This suggests that unacylated ghrelin might fine-tune food intake depending on body condition.

Previous studies had shown a functional correlation between the nocturnal peak of melatonin and *Zugunruhe* in garden warblers and blackcaps (59, 60). Although melatonin did not affect *Zugunruhe* in spring (13), it suppressed *Zugunruhe* in autumn (61). Therefore, it could be expected that melatonin and ghrelin interact in controlling the behavioral and physiological phenotypes of stopover. Evidence for interactions between these two hormones is scarce in birds but has been described in other vertebrates. For example, exogenous melatonin suppresses circulating ghrelin in rats (62) and inhibits ghrelin mRNA expression in the brain of zebrafish (63). The control appears to be bidirectional, because ghrelin reduces melatonin production in the pineal glands of ewes (64). Interactions between ghrelin and melatonin may not be surprising, given that the transition between stopover and migration requires a complete rearrangement of a bird's physiology. Although melatonin might participate in thermoregulation and control of diel rhythms, ghrelin appears to act as a link between fat stores and brain areas that control food intake and locomotor activity. In mammals, GHS-Ra receptors are expressed in the *substantia nigra*, an area known to be involved in the control of locomotor activity (65).

In future studies, it will be interesting to explore if the different forms of ghrelin can influence other hormonal factors that are known to modulate metabolism during migration, such as glucocorticoids. Exogenous acylated ghrelin has been found to cause an increase in corticosterone concentrations in chickens (43). Because corticosterone is elevated in birds ready for departure (66–70), an interplay between these two hormonal systems appears very likely. Also, we need to study the relationship between ghrelin and lipid catabolism—that is, by measuring both ghrelin and the respiratory quotient and by manipulating ghrelin and studying its effect on the respiratory quotient in migratory birds.

Ghrelin is not the only hormone involved in the regulation of food intake, fat storage, and energy mobilization in birds. The set of appetite-stimulating or -suppressing hormones that have been described in mammals have also been found in birds, but their function is far from being understood. The role of leptin in birds has been hotly debated in the past (71–74), and the presence of this hormone in the avian circulation has not been convincingly demonstrated (71, 75–77). Adiponectin, orexin, and obestatin have been studied only at the level of gene cloning or gene expression, but few functional data are available (78–82). Thus, the present study represents a break-through with regard to the role of appetite-stimulating or -suppressing hormones as physiological modulators of stopover decisions during migration. Further, the results of this study are a milestone of migration physiology research, because they represent experimental evidence of specific hormonal modulation of migratory behavior during spring migration. In their comprehensive review, Wingfield et al. (83) wrote in 1990 that "food availability can have pronounced influences on whether an individual migrates that day or rests and replenishes fat stores, etc. The hormonal mechanisms underlying these processes have only been studied superficially." In fact, apart from a previous study showing that increasing melatonin during autumn migration suppresses *Zugunruhe* in Sylvia warblers (61), no clear association between any hormone and migratory behavior or *Zugunruhe* has been demonstrated so far. A number of studies reported contrasting effects of other hormones, such as thyroid hormones, on migratory disposition and/or *Zugunruhe*. However, in most cases, such associations were linked to either very specific treatment schedules or to the life cycle periods before or after migration (83). The strength of our results lies on the experimental approach based on temporary short-term captivity of birds undergoing actual migration and on large sample sizes, which allow us to control for confounding effects such as body condition. Further studies will be necessary to improve our understanding of the role of ghrelin and other hormones linked to the gastrointestinal tract in controlling decision making during migration.

Besides its immediate relevance for bird migration research, our study has important translational aspects. Migratory birds are quite unique in their capacity of rapidly changing body mass and in particular their fat stores when alternating migratory flights and refueling stopovers. However, once they have reached their wintering or breeding areas, they regain a stable (premigratory) lean body mass. Thus, migratory birds are an exquisite model to understand regulation of food intake, fat storage, and lean body mass maintenance. Knowledge gained on the ghrelin system of birds can be used to formulate specific hypotheses on the regulation of food intake, fat storage, and locomotor activity in mammalian models of obesity and to improve our understanding of eating disorders and metabolic syndromes in humans.

Methods

The study was conducted on the island of Ponza (Italy) located in the Tyrrhenian Sea (40°55′N, 12°58′E), which receives large numbers of migratory songbirds during spring migration. When arriving on this island, the birds have just crossed the Mediterranean Sea, a major ecological barrier for songbirds in the Western Palearctic. Garden warblers were caught with mist-nests, and following the ringing standards of the European Union for Bird Ringing, we scored their s.c. fat on a 0–8 scale and the size of the pectoral muscles on a 0–3 scale and measured body mass and the length of the third primary (84). From the body mass and the third primary data we calculated the scaled BMI following ref. 85 as a measure of condition. The scaling exponent (b_{sma}) and the mean length of the third primary necessary for this analysis were calculated from 11,470 garden warblers captured on Ponza between 2012 and 2014.

In a first study, we measured circulating concentrations of ghrelin from samples of garden warblers taken immediately after capture (6:00–13:00 h). Two forms of ghrelin are found in the circulation in vertebrates, which differ for the acylation of the peptide at the third amino acid by n-octanoic, n-decanoic acids, or an unsaturated form of these fatty acids (86). To date, no methods are available to measure unacylated ghrelin in birds; therefore, we focused on acylated ghrelin. Blood samples (150 μL) were collected within 5 min after birds hit the net with Na-heparinized capillaries, and the latency between capture and plasma collection was recorded. Samples were immediately centrifuged, and the amount of plasma was measured with a Hamilton syringe. Then, we added 1 M HCl at a ratio of 1/10 of the plasma volume to stabilize acylated ghrelin. Plasma was then kept on ice and transferred to –20 °C within 1 h.

In a second study, we experimentally tested whether manipulations of acylated or unacylated ghrelin concentrations affected food intake and *Zugunruhe* of garden warblers. Birds were caught between 07:00 and 11:00 h; morphology, fat, and muscle scores were measured as described above; and by 12:00 h all birds were placed into individual, custom-built fabric cages so that they were visually isolated from each other. Each cage was equipped with an infrared activity sensor connected with an activity recorder, which registered locomotor activity within the cage in 2-min intervals (8, 14, 61), and drinking water in a bowl. The room received natural illumination from a large window.

Because we could host only 24 animals each night, we ran the acylated ghrelin and the unacylated ghrelin in two separated years, between the end of April and the middle of May (from May 4–12 the first year; from April 30–May 14 the second year). Each night a similar number of birds was assigned to each treatment, and the experimental groups were staggered across the 2-wk experimental period. This allowed us to achieve large sample sizes. In addition, because weather conditions, the assistants handling the birds, the number of captures per day, and the condition and previous history of birds caught varied substantially during the experimental period, our approach allowed us to account for random variability introduced by environmental factors, subjects' physiological state, and other day or seasonally related factors.

In the first year, we injected 119 birds intraperitoneally with 50 μL 0.9% NaCl saline, 2 μmol/L, or 10 μmol/L recombinant unacylated chicken ghrelin

(Bachem; sequence, H–Gly–Ser–Ser–Phe–Leu–Ser–Pro–Thr–Tyr–Lys–Asn–Ile–Gln–Gln–Gln–Lys–Asp–Thr–Arg–Lys–Pro–Thr–Ala–Arg–Leu–His–OH trifluoroacetate salt) in 0.9% NaCl saline. These injections yielded doses of 100 and 500 pmol per bird, respectively, and were based on previous work involving manipulation of ghrelin levels in domestic birds (30, 31, 38, 87). After receiving the injections at 13:00, the birds were put back into their respective cages and received 6 g of a mixture of dry insect food, boiled egg, and banana (8, 13). Activity was recorded until 7:00 h the next morning, when body mass, fat, and muscle scores of the birds were measured again and released. The remaining food (including what had been scattered around the cage by the birds) was weighed after releasing the birds. Food intake was then calculated by subtracting the weight of the remaining food from the weight at the beginning of the experiment controlling for evaporative water loss measured as the difference in weight of a control food bowl kept in an empty cage for the same period.

In the second year, following the same experimental design described above, we injected 119 birds intraperitoneally with 50 μL of either 0.9% NaCl saline, 2 μmol/L, or 10 μmol/L recombinant acylated chicken ghrelin (Phoenix Pharmaceuticals Inc.; sequence, Gly–Ser–Ser(O-n-Octanoyl)–Phe–Leu–Ser–Pro–Thr–Tyr–Lys–Asn–Ile–Gln–Gln–Gln–Lys–Asp–Thr–Arg–Lys–Pro–Thr–Ala–Arg–Leu–His) in 0.9% NaCl saline. These injections yielded doses of 100 and 500 pmol per bird, respectively, and were based on previous work showing that similar doses resulted in, respectively, low and high physiological circulating levels of ghrelin (30, 31, 38, 87). After receiving the injections, the birds were put back into their respective cages and received 3 g of food (mealworms). We decided to change the food compared with the first year because weighing the scattered wet food was very time-consuming, and in addition, we were concerned that evaporation and wetting of the scattered food by feces and water could introduce additional variability in the measurement. Activity was recorded until 7:00 h the next morning, when the birds were measured again and released. The remaining worms were weighed after releasing the birds.

Because the experiments with unacylated and acylated ghrelin were run in different years, we compared the nocturnal activity of all control animals from both years to test for the presence of a year effect, controlling for condition (scaled BMI). In the first year, the average nocturnal activity (including the 95% credible interval in parentheses) was 0.42 (0.30–0.54) ($\log_{10}$ of *Zugunruhe*), whereas in the second year it was 0.50 (0.40–0.61). Thus, the respective 95% credible intervals of each group overlapped with the mean estimates of the respective other group, demonstrating that there was no systematic difference between the groups and thus no year effect. As expected, birds in better condition showed a larger amount of *Zugunruhe* [$\log_{10}$ slope of condition, 0.21 (0.08–0.34); $F_{3,\ 101} = 7.99$; adjusted $R^2 < 0.17$].

Plasma ghrelin levels were measured using an RIA that has been described previously (42). Acidified plasma was validated to use directly for the RIA by serial dilution of the plasma without extraction similar to tilapia (88). A primary antibody that recognizes the N-terminal portion of octanoylated rat ghrelin (Gly1–Arg11) was used at a final dilution of 1/5,000,000. Octanoylated chicken ghrelin (chicken ghrelin-26-C8), synthesized at Daiichi Suntory Pharma Co. Ltd., Institute for Medicinal Research and Development (Gunma, Japan), was used as the standard peptide instead of rat ghrelin. 125I-(Tyr29)-rat ghrelin was used as a tracer (15,000 cpm per tube). The intra- and interassay coefficients of variation were 4.9% and 5.0%, respectively. The assay had been adapted to allow the determination of ghrelin concentrations from individual samples of small songbirds (sensitivity, 0.5 fmol per tube).

Statistical Analyses. Statistical analyses were conducted with R version 3.2.2 (R Development Core Team 2015) and a Bayesian statistical approach using the packages "arm" (89) and "lme4" (90). We used a general linear model (GLM) to relate the concentration of acylated ghrelin to the fat class controlling for the time between capture and blood sampling. The effect of acylated and unacylated ghrelin on *Zugunruhe* was analyzed with a GLM using the scaled BMI of birds as a covariate. Similar analyses were conducted using fat as covariate (see Tables S1–S5). To obtain unbiased factorial estimates, we scaled and centered the covariates.

Model residuals were analyzed using graphical methods (i.e., qq plots of residual fitted values versus residuals) for homogeneity of variance, violation of normality assumptions, or other departures from model assumptions and model fit. For inferences from the models, we obtained Bayesian posterior parameter estimates and their 95% credible intervals, using the function sim (running 10,000 simulations) and an uninformed prior distribution (91). Unlike null-hypothesis testing, Bayesian statistics do not provide *P* values. Instead, meaningful differences between groups can be assessed by comparing the ranges of the 95% credible intervals between groups. The 95% credible interval provides an estimate for the group mean with a probability of 0.95. If the credible interval of one group does not overlap with the mean estimate of another group, the groups can be assumed to differ from each other. Data are presented as individual data points in combination with Bayesian posterior means and their respective 95% credible intervals (reported in parentheses).

Ethical Statement. All procedures were conducted in respect to the Italian laws and under authorization of the relevant authorities (Regione Lazio, Permit N. B1584 of 21.04.2009, and Permit N. GO2278 of 06.03.2015), and were communicated to the ethical commission of the University of Veterinary Medicine, Vienna.

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1. Alerstam T, Lindstroem A (1990) Optimal bird migration. The relative importance of time, energy and safety. *Bird Migration. The Physiology and Ecophysiology*, ed Gwinner E (Springer, Berlin), pp 331–351.
2. Hedenström A (2008) Adaptations to migration in birds: Behavioural strategies, morphology and scaling effects. *Phil Trans R Soc London B Biol Sci* 365(1490):287–299.
3. Biebach H (1985) Sahara stopover in migratory flycatchers: Fat and food affect the time program. *Experientia* 41:695–697.
4. Biebach H, Friedrich W, Heine G (1986) Interaction of bodymass, fat, foraging and stopover period in trans-Sahara migrating passerine birds. *Oecologia* 69:370–379.
5. Bairlein F (1985) Body weights and fat deposition of palaearctic passerine migrants in the central Sahara. *Oecologia* 66:141–146.
6. Bairlein F (1987) Nutritional requirements for maintenance of body weight and fat deposition in the long-distance migratory garden warbler, *Sylvia borin* (Boddaert). *Comp Biochem Physiol A* 86(2):337–347.
7. Bairlein F (1992) Recent prospects on trans-Saharan migration of songbirds. *Ibis* 134: 41–46.
8. Fusani L, Cardinale M, Carere C, Goymann W (2009) Stopover decision during migration: Physiological conditions predict nocturnal restlessness in wild passerines. *Biol Lett* 5(3):302–305.
9. Goymann W, Spina F, Ferri A, Fusani L (2010) Body fat influences departure from stopover sites in migratory birds: Evidence from whole-island telemetry. *Biol Lett* 6(4): 478–481.
10. Eikenaar C, Schläfke JL (2013) Size and accumulation of fuel reserves at stopover predict nocturnal restlessness in a migratory bird. *Biol Lett* 9(6):20130712.
11. Deppe JL, et al. (2015) Fat, weather, and date affect migratory songbirds' departure decisions, routes, and time it takes to cross the Gulf of Mexico. *Proc Natl Acad Sci USA* 112(46):E6331–E6338.
12. Sjoberg S, et al. (2015) Weather and fuel reserves determine departure and flight decisions in passerines migrating across the Baltic Sea. *Anim Behav* 104:59–68.
13. Fusani L, Cardinale M, Schwabl I, Goymann W (2011) Food availability but not melatonin affects nocturnal restlessness in a wild migrating passerine. *Horm Behav* 59(1):187–192.
14. Lupi S, Goymann W, Cardinale M, Fusani L (2016) Physiological conditions influence stopover behaviour of short-distance migratory passerines. *J Ornithol* 157:583–589.
15. McWilliams SR, Guglielmo C, Pierce B, Klaassen M (2004) Flying, fasting, and feeding in birds during migration: A nutritional and physiological ecology perspective. *J Avian Biol* 35:377–393.
16. Piersma T, Pérez-Tris J, Mouritsen H, Bauchinger U, Bairlein F (2005) Is there a "migratory syndrome" common to all migrant birds? *Ann N Y Acad Sci* 1046:282–293.
17. Landys MM, et al. (2005) Metabolic profile of long-distance migratory flight and stopover in a shorebird. *Proc Biol Sci* 272(1560):295–302.
18. Furuse M, Yamane H, Tomonaga S, Tsuneyoshi Y, Denbow DM (2007) Neuropeptidergic regulation of food intake in the neonatal chick: A review. *Jpn Poult Sci* 44:349–356.
19. Valassi E, Scacchi M, Cavagnini F (2008) Neuroendocrine control of food intake. *Nutr Metab Cardiovasc Dis* 18(2):158–168.
20. Volkoff H, et al. (2005) Neuropeptides and the control of food intake in fish. *Gen Comp Endocrinol* 142(1-2):3–19.
21. Zhang Y, et al. (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* 372(6505):425–432.
22. Kuo AY, Cline MA, Werner E, Siegel PB, Denbow DM (2005) Leptin effects on food and water intake in lines of chickens selected for high or low body weight. *Physiol Behav* 84(3):459–464.
23. Kaiya H, Darras VM, Kangawa K (2007) Ghrelin in birds: Its structure, distribution and function. *Jpn Poult Sci* 44:1–18.
24. Kaiya H, Furuse M, Miyazato M, Kangawa K (2009) Current knowledge of the roles of ghrelin in regulating food intake and energy balance in birds. *Gen Comp Endocrinol* 163(1-2):33–38.

25. Kaiya H, Kangawa K, Miyazato M (2013) Update on ghrelin biology in birds. *Gen Comp Endocrinol* 190:170–175.
26. Kaiya H, Kangawa K, Miyazato M (2013) What is the general action of ghrelin for vertebrates? Comparisons of ghrelin's effects across vertebrates. *Gen Comp Endocrinol* 181:187–191.
27. Kojima M, Kangawa K (2005) Ghrelin: Structure and function. *Physiol Rev* 85(2): 495–522.
28. Thompson NM, et al. (2004) Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 145(1):234–242.
29. Tschöp M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. *Nature* 407(6806):908–913.
30. Geelissen SME, et al. (2006) Peripheral ghrelin reduces food intake and respiratory quotient in chicken. *Domest Anim Endocrinol* 30(2):108–116.
31. Buyse J, et al. (2009) Ghrelin modulates fatty acid synthase and related transcription factor mRNA levels in a tissue-specific manner in neonatal broiler chicks. *Peptides* 30(7):1342–1347.
32. Higgins SC, Gueorguiev M, Korbonits M (2007) Ghrelin, the peripheral hunger hormone. *Ann Med* 39(2):116–136.
33. Wren AM, et al. (2001) Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 86(12):5992–5995.
34. Wren AM, et al. (2001) Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50(11):2540–2547.
35. Furuse M, et al. (2001) Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. *Neurosci Lett* 301(2):123–126.
36. Saito ES, et al. (2005) Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. *Regul Pept* 125(1-3):201–208.
37. Saito ES, et al. (2002) Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks. *Eur J Pharmacol* 453(1):75–79.
38. Shousha S, et al. (2005) Different effects of peripheral and central ghrelin on regulation of food intake in the Japanese quail. *Gen Comp Endocrinol* 141(2):178–183.
39. Haqq AM, et al. (2003) Serum ghrelin levels are inversely correlated with body mass index, age, and insulin concentrations in normal children and are markedly increased in Prader-Willi syndrome. *J Clin Endocrinol Metab* 88(1):174–178.
40. Cummings DE, Frayo RS, Marmonier C, Aubert R, Chapelot D (2004) Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. *Am J Physiol Endocrinol Metab* 287(2):E297–E304.
41. Farner DS (1958) The annual stimulus for migration: Experimental and physiologic aspects. *Recent Studies in Avian Biology*, ed Union AO (Univ of Illinois Press, Urbana, IL), pp 198–237.
42. Kaiya H, Saito E-S, Tachibana T, Furuse M, Kangawa K (2007) Changes in ghrelin levels of plasma and proventriculus and ghrelin mRNA of proventriculus in fasted and refed layer chicks. *Domest Anim Endocrinol* 32(4):247–259.
43. Ocłoń E, Pietras M (2011) Peripheral ghrelin inhibits feed intake through hypothalamo-pituitary-adrenal axis-dependent mechanism in chicken. *J Anim Feed Sci* 20:118–130.
44. Hosoda H, Kojima M, Matsuo H, Kangawa K (2000) Ghrelin and des-acyl ghrelin: Two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun* 279(3):909–913.
45. Akamizu T, et al. (2005) Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. *J Clin Endocrinol Metab* 90(1):6–9.
46. Soares JB, Leite-Moreira AF (2008) Ghrelin, des-acyl ghrelin and obestatin: Three pieces of the same puzzle. *Peptides* 29(7):1255–1270.
47. Hosoda H, et al. (2004) Optimum collection and storage conditions for ghrelin measurements: Octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. *Clin Chem* 50(6):1077–1080.
48. Satou M, Nishi Y, Yoh J, Hattori Y, Sugimoto H (2010) Identification and characterization of acyl-protein thioesterase 1/lysophospholipase I as a ghrelin deacylation/lysophospholipid hydrolyzing enzyme in fetal bovine serum and conditioned medium. *Endocrinology* 151(10):4765–4775.
49. Ariyasu H, et al. (2002) Delayed short-term secretory regulation of ghrelin in obese animals: Evidenced by a specific RIA for the active form of ghrelin. *Endocrinology* 143(9):3341–3350.
50. Toshinai K, et al. (2001) Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochem Biophys Res Commun* 281(5):1220–1225.
51. Patterson M, Murphy KG, le Roux CW, Ghatei MA, Bloom SR (2005) Characterization of ghrelin-like immunoreactivity in human plasma. *J Clin Endocrinol Metab* 90(4):2205–2211.
52. Chen CY, et al. (2005) Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. *Gastroenterology* 129(1):8–25.
53. Asakawa A, et al. (2005) Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* 54(1):18–24.
54. Toshinai K, et al. (2006) Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* 147(5):2306–2314.
55. Tachibana T, Tanaka M, Kaiya H (2011) Central injection of des-acyl chicken ghrelin does not affect food intake in chicks. *Gen Comp Endocrinol* 171(2):183–188.
56. Banks WA, Tschöp M, Robinson SM, Heiman ML (2002) Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 302(2):822–827.
57. Kitazawa T, Hiraga T, Teraoka H, Yaosaka N, Kaiya H (2015) Correlation of ghrelin concentration and ghrelin, ghrelin-O-acetyltransferase (GOAT) and growth hormone secretagogue receptor 1a mRNAs expression in the proventriculus and brain of the growing chicken. *Peptides* 63:134–142.
58. Boswell T, Dunn IC (2015) Regulation of the avian central melanocortin system and the role of leptin. *Gen Comp Endocrinol* 221:278–283.
59. Fusani L, Gwinner E (2001) Reduced amplitude of melatonin secretion during migration in the blackcap (*Sylvia atricapilla*). *Perspectives in Comparative Endocrinology: Unity and Diversity*, eds Goos HJT, Rastogi RK, Vaudry H, Pierantoni R (Menduzzi Editore, Sorento, Italy), pp 295–300.
60. Fusani L, Gwinner E (2004) Simulation of migratory flight and stopover affects night levels of melatonin in a nocturnal migrant. *Proc Biol Sci* 271(1535):205–211.
61. Fusani L, Coccon F, Rojas Mora A, Goymann W (2013) Melatonin reduces migratory restlessness in Sylvia warblers during autumnal migration. *Front Zool* 10(1):79.
62. Mustonen AM, Nieminen P, Hyvärinen H (2001) Preliminary evidence that pharmacologic melatonin treatment decreases rat ghrelin levels. *Endocrine* 16(1):43–46.
63. Piccinetti CC, et al. (2010) Appetite regulation: The central role of melatonin in *Danio rerio*. *Horm Behav* 58(5):780–785.
64. Zieba DA, Kirsz K, Molik E, Romanowicz K, Wojtowicz AK (2011) Effects of orexigenic peptides and leptin on melatonin secretion during different photoperiods in seasonal breeding ewes: An in vitro study. *Domest Anim Endocrinol* 40(3):139–146.
65. Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK (2006) Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol* 494(3):528–548.
66. Landys MM, Ramenofsky M, Guglielmo CG, Wingfield JC (2004) The low-affinity glucocorticoid receptor regulates feeding and lipid breakdown in the migratory Gambel's white-crowned sparrow *Zonotrichia leucophrys gambelii*. *J Exp Biol* 207(Pt 1):143–154.
67. Landys MM, Ramenofsky M, Wingfield JC (2006) Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. *Gen Comp Endocrinol* 148(2):132–149.
68. Landys-Ciannelli MM, Ramenofsky M, Piersma T, Jukema J, Wingfield JC; Castricum Ringing Group (2002) Baseline and stress-induced plasma corticosterone during long-distance migration in the bar-tailed godwit, *Limosa lapponica*. *Physiol Biochem Zool* 75(1):101–110.
69. Lohmus M, Sandberg R, Holberton RL, Moore FR (2003) Corticosterone levels in relation to migratory readiness in red-eyed vireos (*Vireo olivaceus*). *Behav Ecol Sociobiol* 54(3):233–239.
70. Eikenaar C, Bairlein F, Stöwe M, Jenni-Eiermann S (2014) Corticosterone, food intake and refueling in a long-distance migrant. *Horm Behav* 65(5):480–487.
71. Friedman-Einat M, et al. (1999) The chicken leptin gene: Has it been cloned? *Gen Comp Endocrinol* 115(3):354–363.
72. Sharp PJ, Dunn IC, Waddington D, Boswell T (2008) Chicken leptin. *Gen Comp Endocrinol* 158(1):2–4.
73. Amills M, et al. (2003) Identification of three single nucleotide polymorphisms in the chicken insulin-like growth factor 1 and 2 genes and their associations with growth and feeding traits. *Poult Sci* 82(10):1485–1493.
74. Richards MP, Proszkowiec-Weglarz M (2007) Mechanisms regulating feed intake, energy expenditure, and body weight in poultry. *Poult Sci* 86(7):1478–1490.
75. Friedman-Einat M, et al. (2014) Discovery and characterization of the first genuine avian leptin gene in the rock dove (*Columba livia*). *Endocrinology* 155(9):3376–3384.
76. Prokop JW, et al. (2014) Discovery of the elusive leptin in birds: Identification of several 'missing links' in the evolution of leptin and its receptor. *PLoS One* 9(3):e92751.
77. Huang G, Li J, Wang H, Lan X, Wang Y (2014) Discovery of a novel functional leptin protein (LEP) in zebra finches: Evidence for the existence of an authentic avian leptin gene predominantly expressed in the brain and pituitary. *Endocrinology* 155(9):3385–3396.
78. Stuber EF, Verpeut J, Horvat-Gordon M, Ramachandran R, Bartell PA (2013) Differential regulation of adipokines may influence migratory behavior in the white-throated sparrow (*Zonotrichia albicollis*). *PLoS One* 8(6):e59097.
79. Chabrolle C, Tosca L, Crochet S, Tesseraud S, Dupont J (2007) Expression of adiponectin and its receptors (AdipoR1 and AdipoR2) in chicken ovary: Potential role in ovarian steroidogenesis. *Domest Anim Endocrinol* 33(4):480–487.
80. Hendricks GL, 3rd, et al. (2009) Unique profile of chicken adiponectin, a predominantly heavy molecular weight multimer, and relationship to visceral adiposity. *Endocrinology* 150(7):3092–3100.
81. Maddineni S, Metzger S, Ocón O, Hendricks G, 3rd, Ramachandran R (2005) Adiponectin gene is expressed in multiple tissues in the chicken: Food deprivation influences adiponectin messenger ribonucleic acid expression. *Endocrinology* 146(10): 4250–4256.
82. Ocón-Grove OM, Krzysik-Walker SM, Maddineni SR, Hendricks GL, 3rd, Ramachandran R (2008) Adiponectin and its receptors are expressed in the chicken testis: Influence of sexual maturation on testicular ADIPOR1 and ADIPOR2 mRNA abundance. *Reproduction* 136(5):627–638.
83. Wingfield JC, Schwabl H, Mattocks PW (1990) Endocrine mechanisms of migration. *Bird Migration*, ed Gwinner E (Springer, Berlin, Heidelberg), pp 232–256.
84. Bairlein F (1995) *Manual of Field Methods. European-African Songbird Migration Network* (Institute of Avian Research, Wilhelmshaven, Germany), p 32.
85. Peig J, Green AJ (2009) New perspectives for estimating body condition from mass/length data: The scaled mass index as an alternative method. *Oikos* 118:1883–1891.
86. Kaiya H, Miyazato M, Kangawa K, Peter RE, Unniappan S (2008) Ghrelin: A multifunctional hormone in non-mammalian vertebrates. *Comp Biochem Physiol A Mol Integr Physiol* 149(2):109–128.
87. Kaiya H, et al. (2002) Chicken ghrelin: Purification, cDNA cloning, and biological activity. *Endocrinology* 143(9):3454–3463.
88. Riley LG, et al. (2008) Absence of effects of short-term fasting on plasma ghrelin and brain expression of ghrelin receptors in the tilapia, *Oreochromis mossambicus*. *Zoolog Sci* 25(8):821–827.
89. Gelman A, Hill J (2007) *Data analysis using regression and multilevelhierarchical models* (Cambridge Univ Press, New York), Vol 1.
90. Bates D, Mächler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 10.18637/jss.v067.i01.
91. Bolker BM, et al. (2009) Generalized linear mixed models: A practical guide for ecology and evolution. *Trends Ecol Evol* 24(3):127–135.