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PHYSIOLOGICAL FACTORS REGULATING STOPOVER
DECISION IN EUROPEAN MIGRATORY PASSERINES

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General Introduction.....	2
<i>General introduction to migration</i>	<i>2</i>
<i>Physiology of migration in birds</i>	<i>3</i>
Exogenous factors	3
Endogenous factors	4
Hormonal regulation of migration.....	5
Fat as the fuel of migration.....	76
<i>Stopover.....</i>	87
Stopover duration and Zugunruhe	98
Measurements of body condition during stopover	109
Optimal migration theory	11
<i>General introduction to my study</i>	<i>12</i>
<i>General methods.....</i>	<i>15</i>
EURING and ringing activity.....	19
Chapter 1: body condition and stopover decision	20
<i>Introduction.....</i>	<i>20</i>
<i>Material and methods.....</i>	<i>22</i>
<i>Results</i>	<i>24</i>
<i>Discussion</i>	<i>27</i>
Chapter 2: food intake and stopover decision	31
<i>Introduction.....</i>	<i>31</i>
<i>Material and methods.....</i>	<i>33</i>
<i>Results</i>	<i>36</i>
<i>Discussion</i>	<i>41</i>
Chapter 3: ghrelin and stopover decision	43
<i>Introduction.....</i>	<i>43</i>
<i>Material and methods.....</i>	<i>46</i>
<i>Results</i>	<i>51</i>
<i>Discussion</i>	<i>54</i>
General Discussion	57
References	60

General Introduction

General introduction to migration

Movement is an almost universal behavioural characteristic of living organisms. Most movements occur within a relatively circumscribed home range to acquire the resources needed for survival and reproduction. This area including the home range and the required resources is the habitat. During their lifetime, organisms are characterized by different kinds of movement: foraging behaviour, commuting (i.e. albatross foraging), territorial behaviour and ranging (i.e. natal dispersal of birds). Differently, migration takes an individual out of its own habitat to a new one with a new home range often at some distance elsewhere (Dingle & Drake, 2007; Dingle, 1996). Migration occurs across taxa: in insects, birds, many fish, and large mammals, migration is an adaptation evolved to cope with changes in space and time of the environment. Food availability is thought to be a key factor in the evolution of migratory patterns. Despite many studies on migration are confined to a particular group, it is apparent that physiological and behavioural characteristics converge across different taxa, so that migration can be distinguished from other sorts of movements. Migration is a persistent movement not directly responsive to resources or home range and it involves undistracted movements to new habitat. This characteristic has the important consequence that migrants will not end their migration if they encounter favourable resources that are going to deteriorate soon, but they will be especially responsive when they arrive at their ecologically and temporally suitable destination. Consequently, there will be activity patterns particular to departure and arrival. Moreover, during migration animals use external cues such as photoperiod or population density to leave habitats before they deteriorate. Finally, there are specific patterns of energy and internal resource allocation to support movement.

Nowadays, migration is still one of the less understood and most fascinating biological phenomena. Bird migration can be defined as a regular and seasonal movement between the breeding and the non-breeding grounds, which include the return of survivors to the breeding area and it is endogenously controlled (Newton 2008; Salewski & Bruderer 2007). Annual movements of some passerines are impressive: for example, the northern wheatears (*Oenanthe oenanthe*) travel between Alaska and northeast Asia and East Africa, and the willow warblers (*Phylloscopus trochilus*) breed in northeast Asia and winter in Central and East Africa. Migration can be obligate, in that the birds leave their breeding areas at a certain time each year, and return at another time (Newton, 2011). This type of

migration is thus characterised by its regularity, consistency and predictability. In contrast, migration can be facultative, in that the birds may migrate in some years but not in others. This type of migration occurs in response to conditions, such as food availability or weather conditions, at the time. Facultative migration is typical of many partial migrants, and in the so-called irruptive migrants, which typically breed or winter in widely separated areas in different years, wherever conditions are favourable. It is thought that these two types of migration are not distinct, but represent the opposite ends of a continuum of variation in bird migratory behaviour.

Physiology of migration in birds

There are two major aspects of the physiology of migration. The first is the central nervous system (CNS) control of migratory behaviour. This aspect involves not only the active enhancement of locomotor activity, but also the inhibition of feeding and reproduction, and of the responses to the stimuli that would ordinarily trigger those actions. There is evidence from a number of animals that such inhibition occurs, often accompanied by major changes in the gonads or the gastrointestinal tract, for example. Many songbirds migrate at night when they would otherwise sleep, and many shorebirds reduce their gastrointestinal tract which permits them to feed only on easily digestible food (Piersma & Gils, 2011). The second aspect involves the mobilization of hormonal and metabolic pathways to stimulate and regulate migration and support its high energetic costs. The preparation for migration include responses to external inputs, neurosecretory and hormonal action, the organization of energy reserves, metabolic and morphological shifts and storage of suitable fuels.

Exogenous factors

First, organisms must be able to synchronize their migration to the environmental fluctuations, so that they can anticipate changes that will occur. The most common environmental changes are those associated with the seasons and the most reliable predictor is the annual change in day length, as a result of the Earth circles the Sun. Most of the organisms, including migrants, respond to changes in photoperiod. In birds, the response to cues must be co-ordinated with other life functions such as reproduction and molt (Ramenofsky et al. 2012). In spring, the sequence of migration and breeding is stimulated by long days. When photoperiod reaches critical daylength, it enhances release of orexigenic-neuropeptides that induce hyperphagia, followed by fattening. Photoperiodic stimulation of the hypothalamic–pituitary–gonad axis (HPG) results in secretion of gonadal

androgen. This may stimulate feeding, flight muscle hypertrophy, erythropoiesis, and increase migratory disposition (**Figure 1**). As the season progresses, birds become photorefractive. There appears to be carry-over between spring and autumn events with spring activation acting as a “remote timer” to terminate breeding and initiate autumnal events such as fattening and in some species a postnuptial molt (Conklin et al. 2010; Helm & Gwinner 2005). Further, there may be renewed photosensitivity with acceleration of physiological changes by shortening days (Helm et al. 2009).

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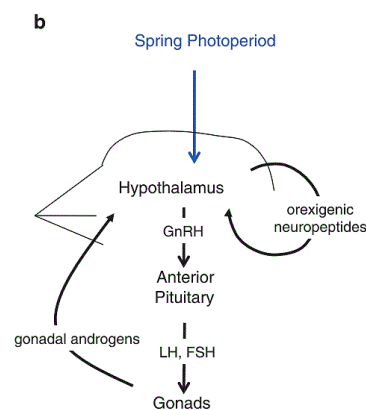


Figure 1 The hypothalamus-pituitary-gonad (HPG) axis in birds. From Ramenofsky et al. 2012

Endogenous factors

In addition to exogenous factors like photoperiod, endogenous timing mechanisms play an important role in migration and its coordination with other associated life-history events such as molt and fattening (Dingle 1996; Gwinner & Helm 2003; Helm et al. 2013; Ramenofsky et al. 2012). The main endogenous factors are rhythmic daily (circadian) and annual (circannual) cycles. When organisms are kept under constant conditions, their circadian and circannual rhythms will “free run” independently of external inputs, although with periods that only approximate a day or a year, thus demonstrating that they are endogenously driven. Rhythms are precise in natural populations because they are synchronized by external *zeitgebers* (“time givers”), in most cases by photoperiod. During the migratory period, migrants enter the so-called migratory disposition (Berthold 1996), characterized by hyperphagia, fat deposition, change of diel pattern of locomotory activity

(nocturnal migrants start to be active at night in addition to their normally daytime activity) and spontaneous orientation in the migratory direction. Migrants taken in captivity develop all these characteristics (Gwinner 1968) and show migratory disposition under constant conditions during many years in captivity (Gwinner 1996), with a period that approximates 12 months (Berthold et al. 1972; Gwinner 1986).

Hormonal regulation of migration

Migration in birds involves several physiological functions with many regulated by hormones (Piersma & van Gils 2011; Wingfield et al. 1990). These functions include (1) fat deposition, (2) enzyme integration for energy store and locomotion, (3) increased hematocrit for enhanced oxygen transport, (4) hypertrophy of flight muscles, (5) reduction in gut mass, and (6) development and synchronization of migratory behaviour. Hormonal regulation of these processes is complex and often not clear.

In spring, increasing daylength stimulates neuroendocrine pathways in the brain of birds (Hahn & MacDougall-Shackleton 2008). These pathways produce gonadotropin-releasing and –inhibiting hormones which are transmitted to the hypothalamus and the pituitary. Both types of hormone influence the secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones induce testosterone production in males, estradiol and progesterone in females. In both sexes, sex steroids feedback to the HPG axis to regulate the gonadotrophic hormones. The consequence of this hormonal cascade is that birds arrive at the breeding grounds in condition to begin reproductive activities shortly after arrival. In addition to the changes in the reproductive system, in spring birds also display hyperphagia, fattening, and migratory restlessness prior to departure. The role of the hormones in generating these responses is still not very clear. The neuroendocrine pathways controlling fattening versus restlessness and gonadal activity are apparently separate, as revealed by electrolytic lesions in specific sites in the hypothalamus (Ramenofsky 2011). It has been speculated that androgens organize feeding centers to promote fattening, but how this organization works and how it may carry over to migration in autumn remains obscure. It is the case, however, that gonadal hormones do stimulate fattening prior to spring migration, as demonstrated by gonadectomy.

Other hormones may also be involved in bird migration. Prolactin has been assumed to stimulate appetite and fattening, but experimental results are unclear and the peak of prolactin production is not coincident with the onset of fattening in either White-crowned

Sparrows (*Zonotrichia leucophrys*) or Dark-eyed Juncos (*Junco hyemalis*) (Holberton et al. 2008; Ramenofsky 2011). There is increasing evidence that corticosterone (CORT) plays an important role in behavioural and metabolic processes during migration itself (Holberton et al. 2008; Landys et al. 2006; Landys-Ciannelli et al. 2002). CORT is a stress hormone that promotes fat metabolism and provides glucose to target tissues. It may play at least two roles during migratory flight: (1) to mobilize stored fuels needed for flight, especially if unpredictable (metabolically stressful) conditions are encountered; and (2) to sustain the availability of fuel for target tissues including flight muscles, heart and brain. Additional mobilization of activity may involve thyroid hormones such as T₃ and T₄. Furthermore, leptin may be involved in bird migration. In mammals and several non-mammalian species, leptin is synthesized primarily by adipose tissue and its circulating concentration is proportional to total body adiposity (Crespi & Denver 2006; Londraville & Duvall 2002; Niewiarowski et al. 2000; Ostlund et al. 1996). Leptin provides a signal to the brain through hypothalamic receptors, triggering the regulation of lipid metabolism, the reduction of food intake and increase in energy expenditure. Although the existence of this hormone in birds is still controversial, there is an abundance of evidence that a leptin functional receptor and a leptin-like immuno-reactivity does exist in birds (Adachi et al. 2008; Amills et al. 2003; Cassy et al. 2004; Dridi et al. 2005; Dridi et al. 2007; Hen et al. 2008; Kochan et al. 2006; Kordonowy et al. 2010; Ohkubo et al. 2014; Prokop et al. 2014). Hence, leptin could have a role in the control of migration by regulating the availability and amount of fuels that are used to power migratory journeys, and thus influencing decisions involved in stopover timing and behavior (Cerasale et al. 2011).

Another excellent candidate that might be involved in bird migration is the recently discovered hormone ghrelin, produced by the gastrointestinal tract. It appears to have an important regulatory function. In domestic galliforms, ghrelin reflects the nutritional state (fasted or fed) and food availability (lipid sensor function) (Richards & McMurtry 2010), acts as an anorexic hormone in regulating glucose metabolism (Kaiya et al. 2009; Kaiya et al. 2013), with peripheral anti-lipogenic effect (Buyse et al. 2009; Geelissen et al. 2006). Hence, ghrelin may trigger the behavioural and metabolic switches between stopovers and migration.

Fat as the fuel of migration

The metaphor of marathon running is inadequate to fully capture the magnitude of long-distance migratory flight of birds...a journey to the moon seems more appropriate.

(Guglielmo 2010, p.336)

Mammals fuel primary with glycogen stored in muscle cells. Migrant birds, however, store fat, and fuel their migratory flights primarily through fatty acid metabolism (McWilliams et al. 2004; Pierce et al. 2005). In fact, fat has great advantages (Dingle 1996; Piersma & Gils 2011). When oxidized, 1 g of fat yields 38 J of energy compared with 5 J for wet protein and 4 J for wet carbohydrate. This is largely because fat does not require water for storage; each gram of carbohydrate (as glycogen), in contrast, requires an additional 3 g of water. Even without water, fat is more than twice as energy rich as “dry” carbohydrate or protein (Jenny & Jenni-Eiermann 1998; Piersma & Gils 2011). Furthermore, maintenance costs for adipose tissue are also 10 times less than for other tissues. Before migrating, birds can store enormous amounts of fat. Many migratory songbirds double their body weight by depositing pre-migratory fat. Even though a bird with 50% body fat consumes about 40% more energy than a bird covering the same distance with only a 10% fat load, it can fly for three to four days up to about 4000 km (Bruderer 1992). The insolubility of the component fatty acids means that they are difficult to transport to working muscles at a rate fast enough to support the aerobic demands of flight (Guglielmo 2010). Birds compensate for this by expressing large amounts of fatty acid transport proteins on muscle membranes and in the cytosol.

The accumulation of the energy reserves occurs by means of diets shifts and modification of metabolic pathways (Berthold et al. 2003; Dingle 1996). First, pre-migrants greatly increase their appetites and this hyperphagia involves more rapid rates of feeding. Second, accompanying enhanced appetite are increases in lipid synthesis and rapid deposition of fat in subcutaneous “depots”. This hyperlipogenesis depends not only on the increased food intake, but also on increased rate of fatty acid synthesis in the liver. The third contributing factor is the diet shift. For example, observations on a number of species in both the Old and New Worlds have noted that autumn migrants feed heavily on fruits (Franz Bairlein 1990; Peter Berthold et al. 2003; Dingle 1996).

Stopover

Most of the migrants do not cover the distance between their breeding and wintering quarters by a single flight, but by multiple stopovers *en route* during which they rest and refuel (Chernetsov 2012). Migratory stopover is necessary for refuelling and/or because continuing migration is temporarily too expensive or challenging because of external factors such as adverse weather conditions, e.g. high air temperature causing negative water balance, headwind, low visibility, precipitation etc. (Biebach 1990; Biebach et al. 1991, 2000; Dolnik 1990; Schmaljohann et al. 2007). In nocturnal migrants, flights and stopovers are usually well separated. When migrating over areas with continuous stopover possibility, i.e. when not crossing ecological barriers, birds migrate at night and rest and forage during daytime. Only when nocturnal migrants are facing large water bodies or other areas with very poor stopover opportunities, their pattern of flight activity changes so that they can perform both diurnal and nocturnal migration. For example, it happens in small European passerines when crossing the Sahara Desert and the Mediterranean Sea, more frequently in spring than in autumn (Adamik et al. 2016; Schmaljohann et al. 2007). Songbirds can stop over at any sites along their flyway if they are migrating over optimal suitable habitats. This is called a continuous stopover opportunity as opposed to a discrete stopover opportunity (Houston 1998). The role of stopovers is fundamental in terms of both time and energy. Actual flight represents about 30% of energy expenditure during migration, whereas the rest is spent on supporting the animals during stopovers (Wikelski et al. 2003). This proportion is predicted by the optimal migration theory (Alerstam & Hedenström 1998). As stopovers play a determinant role in temporal and energetic balance of migration, their significance for organization of migration is primary. The main ecological parameters of a migratory stopover are its duration, fuel deposition rate and fuel load at departure. *Fuel deposition rate* (FDR) is the rate of body mass change in a migrant during stopover. It may be measured in grams per hour or grams per day, or as a percentage of the initial or lean body mass per hour or per day. Even though the word 'deposition' implies mass gain, FDR may be either positive if a bird is gaining body mass, or negative if it loses mass. *Departure fuel load* is the amount of fuel (i.e. energy) stored in the form of extra mass, which typically consists of lipids and proteins, deposited above the lean body mass at migratory departure. These parameters are interrelated and they are studied by the optimal migration theory (Alerstam & Hedenström 1998; Alerstam & Lindström 1990; Alerstam 2011; Houston 1998; Weber & Houston 1997).

Stopover duration and Zugunruhe

Duration of migratory stopovers of songbirds usually varies between 1 and 15 days. Sometimes, especially before and just after crossing large ecological barriers, stopovers may reach 20-25 days. Significant proportions of migrants stop over for only one day and resume migration on the first night after arrival.

Several methods are used to measure stopover duration. Using the capture-recapture method, that means catching stopping migrants and trying to recapture or resight them, is common. This method provides the *minimum stopover length*, that is time in days or hours elapsed between the first capture of a bird and its last capture or resighting. The most objective method of estimating stopover duration is radio-tagging of migrants by small VHF transmitters. This method allows tracking of individual migrants until their migratory departure. However, the reception range of the transmitters is usually not very large (400-1500 m). As birds can undertake exploratory flights or landscape-scale movements for up to 1.5-2 km during daytime (Chernetsov & Mukin 2006; Fransson et al. 2008) and this behaviour may result in the loss of signal (Mills et al. 2011; Schmaljohann et al. 2011), it is necessary to do research on small islands where movement opportunities are limited (Aborn & Moore 1997; Goymann et al. 2010; Schmaljohann et al. 2011). Finally, an indirect measure of stopover duration can be obtained through the recording of migratory restlessness, or Zugunruhe. In fact, migratory birds held in captivity show Zugunruhe during migratory periods: they hop and flit back and forth in their cages, and flutter their wings at high frequency but low amplitude ('whirring') while sitting on their perches. Zugunruhe is particularly obvious in nocturnal migrants. Zugunruhe was interpreted as an indicator of the migratory urge of birds, and Naumann (1795-1817) was probably the first to hypothesize that the amount of Zugunruhe might reflect the length of the migratory period and migration distance. Zugunruhe, especially for nocturnal migrants, is easily registered quantitatively in so-called registration cages. The most common method employed in past studies was the use of mobile perches connected to microswitches. Nowadays, infrared sensors, ultrasound, or air-pressure switches have replaced the microswitches. Results of studies carried out on over 100 species demonstrated that the amount of nocturnal activity of caged migrants is well correlated with the length and sequence of the migratory journey of their free-living conspecifics, suggesting that Zugunruhe of captive migrants is a correct manifestation of the flight activity in the wild (Berthold 1996; Berthold 1973).

Measurements of body condition during stopover

As birds fuel their migratory flights primarily through fatty acid metabolism, the amount of fat fuel is the most important measure of body condition during migration. Beside fat, migrants also use muscle proteins in flight. Hence, the size of the pectoral muscle is a further valuable indicator for body condition in migrants. Finally, the measure of body mass is another fundamental indicator of body condition.

Both fat and muscle score can be assessed visually. The size of the visible fat depot is determined with the use of a 9-grade score (0 - 8) according to Kaiser (Kaiser 1993) (Figure 2). Two of the most important fat deposits are the furcular (intra-clavicular depression) and the abdomen (Bairlein, 1994). These deposits are visible through the skin, allowing a rapid visual assessment of the body condition of the bird. The size of the breast muscle is a further valuable indicator for body condition in migrants (Figure 3). In birds whose flight muscles are not covered by fat the shape of the breast muscles can be easily scored.

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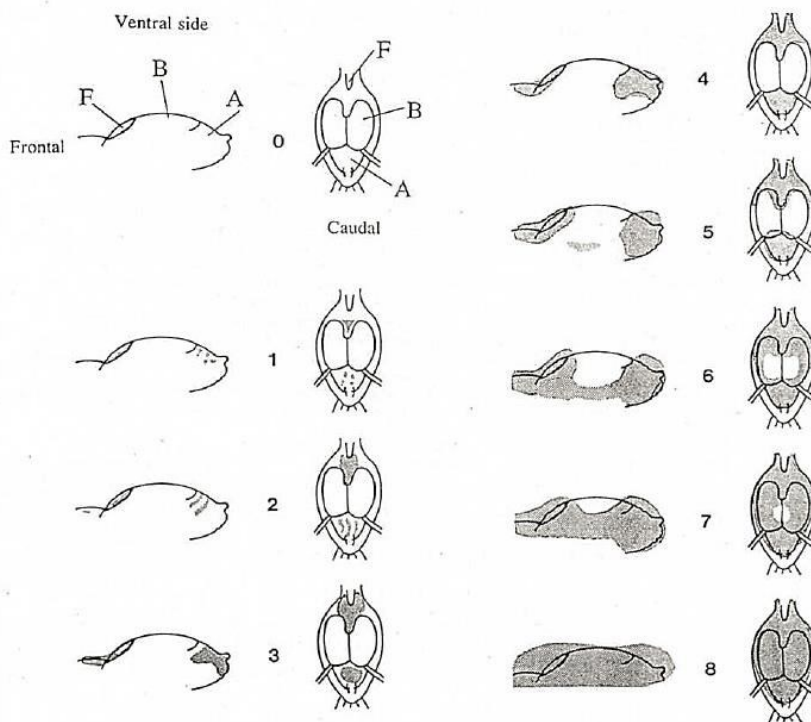


Figure 2 Fat score classes 0-8. Fat = stippled areas. F = furcular depression, B = breast muscle, A = abdomen. From Kaiser 1993, modified

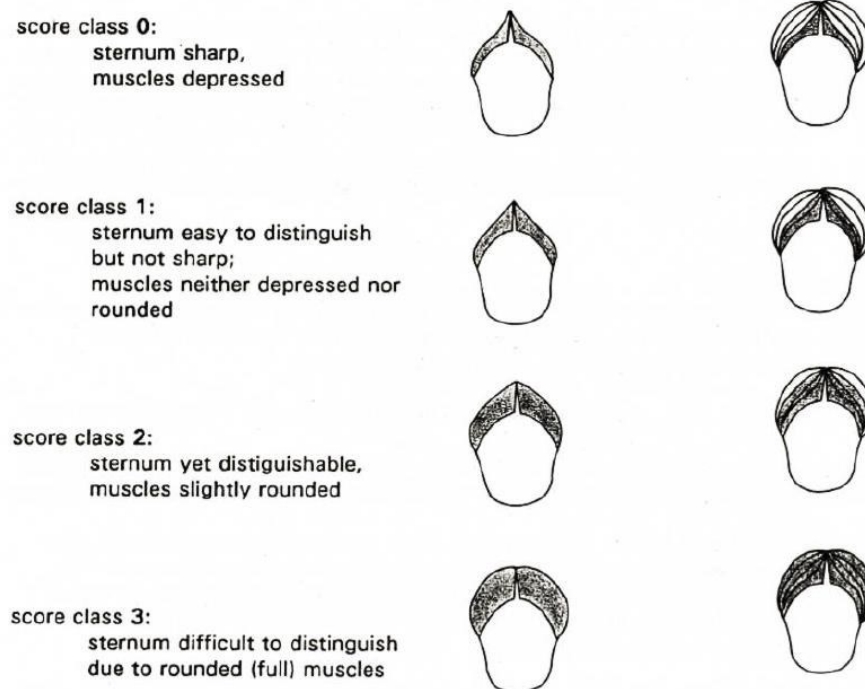


Figure 3 Muscle score. The right column shows the relative difference between the scores. The stippled areas are the muscles. Drawings by Göran Walinder, Falsterbo B.O.

Optimal migration theory

Migrating birds face a number of alternative decisions during migration, such as how fast to fly, where to stop for refuelling and how much fuel to accumulate before departing on the next flight. These decisions are presumably made in relation to rules governed by some overall strategy for the migration to be as fast, energy conserving or as safe as possible (Alerstam & Lindström 1990). Given that the majority of annual mortality occurs during migration (Sillett & Holmes 2002), survival could be maximized by minimizing the overall time of migration and hence the exposure to danger. Alternatively, an economical use of energy could lead to reduced exposure to predation during foraging, or reducing the exposure to food-related parasites, and hence an energy minimization strategy may be optimal (Hedenström 2008).

During migration, bird decisions in terms of stopover duration and feeding/resting behaviour should be optimal according to their migratory pattern and distance to their breeding quarter. Optimization is the process of minimizing costs or maximizing benefits,

or obtaining the best possible compromise between the two. Depending on which currency the birds are optimizing, e.g. time or energy, different optimum fuel loads at departure from a stopover are expected. Hence, by registering departure fuel loads it is possible to infer which currency the birds are optimizing. If birds are mainly selected to minimize the overall time of migration, their departure fuel load will be sensitive to variation in food abundance at stopover and dependent of fuel deposition rate, while minimization of energy cost of transport predicts invariant departure fuel load with respect to fuel deposition rate (Alerstam & Lindström 1990).

General introduction to my study

My research was focused on the physiology of stopover in European migratory passerines during spring migration. I investigated physiological factors that might regulate the decision of birds to prolong the stopover or resume migration. In fact, particularly in the spring, birds are under time constraints to reach their breeding grounds early and get better territories. My main interest was to study the influence of several physiological factors on behaviour and metabolism of migrants during stopover. In particular, I studied the role of 1) body condition, 2) food intake, and 3) orexic-anorexic hormones on stopover decision.

I conducted the fieldwork in Ponza, a small island in the Tyrrhenian Sea (40.855°N, 12.858°E), located along one of the main Mediterranean migratory routes (*Figure 4*~~*Figure 4*~~). In fact, this island is a major stopover site for trans-Mediterranean migratory passerines. Our Research Station has been active since 2006, associated with the Ponza Ringing Station, which has one of the highest net efficiencies in the Mediterranean (about 20,000 captures per season, *Figure 5*~~*Figure 5*~~). Hence, we could rely on very large sample sizes throughout the migratory season (*Figure 6*~~*Figure 6*~~). The Ringing Station, which opened in 2002, is currently involved in the 'Small Island Project'. This project aims to assess the different patterns of the spring migration through the Mediterranean Sea, which represents a wide ecological barrier between Africa and Europe for migratory birds. The main scope of the Small Island Project is tracking the migratory paths and the phenology of the seasonal movements of different species, mainly passerines.

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Figure 4 The position of the island of Ponza



Figure 5 View of the island of Ponza and a mist-net



Figure 6 Garden warbler in a mist-net (photo © Andrea Ferretti)

My study species were all nocturnal migrants, but strongly differed in their migratory pattern. Garden warbler (*Sylvia borin*) is a long-distance migrant that faces long migratory flights over the Sahara Desert and the Mediterranean Sea and reaches its breeding grounds in Northern Europe. On the other side, robin (*Erithacus rubecula*), black redstart (*Phoenicurus ochruros*), and stonechat (*Saxicola torquata*) are short-distance migrants that face shorter flights from Northern Africa to Italy or Central Europe (**Figure 7**).

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Figure 7 Study species: garden warbler (a), robin (b), black redstart (c), stonechat (d)

General methods

We caught the birds with mist-nets during spring migration from March to May, following two general procedures: 1) sampling at the nets and 2) 'overnight stay'.

- 1) The animals were taken immediately after entering the nets to collect a blood sample. The general method of blood sampling must be conducted within 3 minutes after capture in order to avoid a stress hormonal response. In fact, glucocorticoids such as corticosterone generally start to increase 3 minutes after the initiation of an acute stressful stimulus in vertebrates (Wingfield et al, 1997). The method involved the following procedures:
 - Continuous monitoring of the mist nets between 7:30 and sunset, taking care of not disturbing the birds (**Figure 8**)
 - Transferring of the bird to the bleeding station, immediately after the capture

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- Collection of 200 μ l of blood from the wing vein, using G23 needles and Na-Hep covered capillaries and transferring of the blood in Eppendorf tube 1.5 ml (*Figure 9*)
- Immediate centrifuge of the blood sample (5000 rotations per minute for 5 minutes)
- Collection of the plasma (about 70 μ l of plasma in 200 μ l of blood) with Hamilton syringe and transferring of the plasma in Eppendorf tube 0.5 ml
- Immediate addition of 1M HCl at a ratio of 1/10 to the plasma (in case of blood sampling for ghrelin measurement)
- Labelling of the plasma sample
- Temporary storage of the plasma sample in ice
- Storage of the plasma sample in a freezer at -20°C within an hour
- Assessment of the standard physiological measurements, i.e. extent of subcutaneous fat deposit (fat score: 0-8), thickness of pectoral muscles (muscle score: 0-3) and body mass
- Ringing procedure, according to the standard procedures of the European Union for Bird Ringing ('EURING and ringing activity', see paragraph below)
- Release of the bird

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Figure 8 Monitoring of the mist-net (photo © Andrea Ferretti)



Figure 9 Collection of blood samples at our bleeding station, next to the mist-nets

2) In the 'overnight' approach, birds taken from the nets were short-term housed in indoor cages. This approach permitted the study of internal factors avoiding the influence of environmental factors. A special room that receives natural light was equipped with 24 individual custom-made cages (45 x 45 x 60 cm), composed of a metal frame covered by white fabric that allows air circulation while screening the birds from other individuals and the researchers (**Figure 10**~~Figure-10~~). Each cage was equipped with 3 perches and an infrared sensor that records the diurnal activity and the migratory restlessness (Zugunruhe) (**Figure 11**~~Figure-11~~). The system allowed the simultaneous recording of 24 cages. Thus, depending on the capture success rate, up to 24 birds/day were tested simultaneously. The method involved the following procedures:

- Every hour monitoring of the mist nets between 7:30 and 11:30
- Trapping and ringing procedures
- Transferring to a specific room equipped with the individual custom-made cages
- Assessment of the standard physiological measurements
- Housing within 2 hours after capture and by 12:00
- Water *ad libitum* and food supply (or only water, according to the experiment) at the end of the housing operations
- Hormonal treatment at 13:00, if required by the experiment (those birds were given food after the injection)
- Non-stop recording of diurnal and nocturnal locomotory activity
- Assessment of the standard physiological measurements and releasing the following morning at 6:00

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Figure 10 Room equipped with the 24 individual custom-made cages



Figure 11 Individual cage equipped with an infrared sensor and three perches

EURING and ringing activity

As migratory birds cross political borders, international cooperation is fundamental for the study of the phenomenon of migration. The European Union for Bird Ringing (EURING) ensures the coordination and standardization of bird ringing in Europe. The standardized data issued by the ringing activity are useful both for research and management purposes. Indeed, the monitoring of birds supports the studies on dispersion and migration, behavior, longevity and surviving rates, demographic growth and reproductive success. The ringing activity consists of tagging birds with an aluminium ring, which contains the identification number and the address of the central reference institute of the issuing country. Particularly the analysis of recaptured birds provides important information about itineraries, areas of overwintering, migration speed and maximum age reached of migratory birds. Birds are caught with a system of standardized mist-nets. Mist-nets are typically made of nylon or polyester, potentially invisible to birds, and they are suspended between two poles. Mist nets have shelves created by horizontal lines that form a loose, baggy pocket. When a bird hits the net, it falls into this pocket, where it becomes tangled. The nets are operative from the sunrise until the sunset and they are checked every hour or more often (depending on the weather conditions) by expert volunteers of the ringing station. Once released from the net, each bird is put in a separate cotton bag and brought to the ringing station, where the ringer records the following parameters: ring number, sex, age, moult, species, feather length, date and time of capture, wing length, weight, fat score, and muscle score. Birds taken out of the nets during the nets round starting, for example, at 9:00 are recorded in the ringing schedules for 8:00, since they have entered the nets between 8:00 and 9:00. All the data are registered in a database implemented by the 'Istituto Superiore per la Protezione e la Ricerca Ambientale' (ISPRA). The ISPRA has been coordinating the 'Progetto Piccole Isole' (PPI) since 1988. The PPI involves several small islands and coastal sites mostly in the west-central part of the Mediterranean basin. This project aims to assess the different patterns of the spring migration through the Mediterranean Sea, which represents a wide ecological barrier between Africa and Europe for migratory birds. The main scope of the PPI is tracking the migratory routes and the phenology of the movements of different species (mainly passerines), focusing on the long-distance migrants, which winter in areas south of the Sahara Desert. Moreover, the PPI aims to investigate the functional role of the small Mediterranean islands as stopover site. The PPI also provides the rings for the bird tagging.

Chapter 1: body condition and stopover decision

Introduction

Every year billions of birds migrate across areas where food and water are not available, with non-stop flights that lead to a rapid exhaustion of energy reserves. Thus, stopovers for refuelling are necessary to successfully continue migration. The duration of stopovers is influenced by a number of factors including endogenous programmes, environmental parameters such as weather and food availability, and physiological condition of each individual at arrival (Jenny & Shaub 2003). Theory predicts that migratory strategies and stopover behaviour depends on fat deposition (Alerstam & Lindström 1990; Hedenström 2008), but evidence for a determinant role of body condition for stopover duration has been reported only in long-distance migratory passerines (Fusani et al. 2009; Fusani & Gwinner 2004; Goymann et al. 2010). To date, our knowledge about stopover behaviour of short-distance migrants is still scarce.

In captivity, the extent of migratory disposition of nocturnal migrants is estimated by the intensity of nocturnal restlessness, or *Zugunruhe* (Naumann 1795-1817). The duration and intensity of *Zugunruhe* is correlated with the duration and intensity of migration in free-living birds (Berthold 1973). In garden warblers (*Sylvia borin*), it was noted that leaner birds show a diurnal pattern of activity, whereas heavier birds show low diurnal activity and intense nocturnal activity, i.e. *Zugunruhe* (Bairlein 1985). In northern wheatears (*Oenanthe oenanthe*), birds with large fuel loads showed more *Zugunruhe* the night after capture at a stopover site and accumulated less fuel in the subsequent two days than birds caught with small fuel loads (Eikenaar & Schläfke 2013). These observations match with the hypothesis that lean birds would spend more than one day at a stopover site to refuel and recover, whereas fat birds would resume migration in the night following arrival (Biebach 1985). This hypothesis was confirmed by laboratory studies in which a non-stop flight followed by stopover was simulated in fasting-and-refeeding experiments (Biebach 1985; Fusani & Gwinner 2004; Gwinner et al. 1988). In spring, the effects of the food regimen on nocturnal activity during the night following food reintroduction were highly correlated with the amount of fat reserves in blackcaps (*Sylvia atricapilla*). These results were confirmed later by field studies, in which body condition was correlated with the extent of *Zugunruhe* in garden warblers, whitethroats (*Sylvia communis*) and whinchats (*Saxicola rubetra*; Fusani et al. 2009). In particular, we observed that birds with high fat reserves showed intense *Zugunruhe* at night and little activity during the day, whereas

birds with little fat reserves showed the opposite pattern, suggesting that the physiological condition influences stopover duration in European long-distance migrants (Fusani et al. 2009). Furthermore, we confirmed these results in a whole-island telemetry study, in which we demonstrated the influence of fat reserves on real minimum stopover duration (Goymann et al. 2010).

Both long- and short-distance migrants are thought to migrate using a time-minimization strategy (Hedenström 2008). In spring, selection favours early arrival and onset of breeding (Kokko 1999). Several studies suggested that the endogenous circannual mechanisms controlling migratory parameters are particularly well developed in long-distance migrants (Bairlein & Gwinner 1994). Across areas where refuelling stopover sites are available, endogenous factors should dominate the duration and length of migratory flights, whereas condition and extent of energy reserves are expected to determine the stopover duration before crossing an ecological barrier (Jenny & Shaub 2003). Similarly, body condition is likely to be crucial in determining stopover duration after crossing a barrier. In fact, when crossing ecological barriers, birds are usually unable to stop and refuel. Furthermore, some field data suggest that long-distance migrants deposit more fat before migration and also maintain fat reserves longer than short-distance migrants (Bairlein & Gwinner 1994). Therefore, energy reserves are expected to strongly determine the stopover behaviour in short-distance migrants. Previous studies on four different species of North American thrushes, wintering in Central America or in the North of South America and crossing the Gulf of Mexico, showed that lean birds are less active at night than fat birds just after the overcoming of the ecological barrier (Yong & Moore 1993), revealing the priority of birds without fat load to replenish energy reserves.

A few studies had previously demonstrated a simultaneous effect of body condition and weather factors on stopover duration in short-distance migrants (Bulyuk & Tsvey 2013; Morganti et al. 2011). In fact, when weather conditions are favourable (strong tailwinds, weak crosswinds, low rainfall, high temperature, high pressure), fat black redstarts (*Phoenicurus ochruros*) are highly likely to depart from the stopover site on the evening of the same day of arrival (Morganti et al. 2011), and although weather conditions alone do not determine the duration of stopover in robins (*Erithacus rubecula*), birds that stop for longer than one night tend to depart when the wind is favourable (Bulyuk & Tsvey 2013). We thus predict that short-distance migrants rest at the stopover site in relation to their physiological condition, similar to long-distance migrants caught at the same stopover site

(Fusani et al. 2009). Here, we studied the relationship between physiological condition and the amount of Zugunruhe of short-distance migrants after crossing the Mediterranean Sea. Birds were caught at a stop-over site and placed in cloth cages to measure activity for one day and released the following morning. Our results demonstrate that the physiological condition predicts the intensity of Zugunruhe in all three species of short-distance migrants.

Material and methods

The study was conducted on Ponza (Italy), a small island in the Tyrrhenian Sea (40.855°N, 12.858°E) located along one of the main Mediterranean migratory routes. A ringing station has been active on the island since 2002. We used 51 black redstarts (*Phoenicurus ochruros*), 98 robins (*Erithacus rubecula*) and 17 stonechats (*Saxicola torquata*), caught with mist-nets during spring ringing operations in March and April 2011 and 2013. These three species are short-distance migratory passerines, which winter in the Mediterranean coasts of North Africa. Data from capture-recapture studies suggest that in spring robins migrate to central-northern Europe, mainly to Sweden and Baltic coasts, black redstarts migrate to central Europe, mainly to Germany, and stonechats migrate to southern-central Europe, mainly to Italy, Austria and Hungary (Spina & Volponi 2008). Data from the Ponza ringing station show that the peak timing of passage (median Julian day) in the years 2007-2014 ranges from 82 to 97 in robins, 79-94 in black redstart, and 73-81 in stonechats (Cardinale unpublished). Birds were caught in the morning between 7:30 and 11:30, subcutaneous fat was scored on a 0–8 scale, the size of the pectoral muscles on a 0–3 scale, and body mass was measured to the nearest tenth of a gram (Bairlein 1994). By 12:00, birds were placed in individual cloth cages so that they were visually isolated from each other. Each cage was equipped with an activity infrared sensor recording the locomotor activity. Birds were given only water ad libitum and were exposed to natural daylight conditions. All birds were released the following morning within one hour after sunrise.

We calculated the number of times the infrared sensor was activated during each 2 min period. We then calculated the average activity during the intervals from 12.00–sunset (day activity) and from sunset–sunrise (Zugunruhe) based on civil twilight times (Greenwich Mean Time +1). As the activity data were saved every day at 24.00, we calculated the average activity during the intervals sunset-24.00 (Zugunruhe during the first part of the night), and 24.00-sunrise (Zugunruhe during the second part of the night), and we calculated Zugunruhe as the mean value of the two previous variables. Finally, we

transformed all the calculated values of activity (Zugunruhe during the first part of the night, Zugunruhe during the second part of the night and Zugunruhe) by taking the natural logarithms ($\ln(x+1)$). To test for the effects of body condition on Zugunruhe, we performed first an analysis with the fat scores. We chose this approach because the fat score is a normalized index which is directly comparable across species and previous studies have shown it to be a robust, consistent predictor of migratory disposition (Fusani et al. 2009). It was not possible to use GLMs (multiple regression) to study the relationship between fat score and Zugunruhe across species because of the study design, i.e. the species is a categorical variable with multiple levels. Therefore, we used a non-parametric approach. First, we performed a non-parametric Spearman correlation between fat score and Zugunruhe including all species. The effect of the species on the relationship between fat and Zugunruhe was then analysed by means of the Quade's Test, a non-parametric Analysis of Covariance. We also performed separately for each species a Pearson correlation test between Zugunruhe and the index CONDITION, which was extracted by means of principal component analysis (PCA) from body mass, fat score, and muscle score. All statistical analyses were performed with R v. 3.2.0 using a significance level of $\alpha = 0.05$.

Results

A global analysis across the three species considered together, on a total of 167 individuals (51 black redstarts, 99 robins, 17 stonechats), yielded a significant positive correlation between Zugunruhe and fat score ($r_s = 0.263$, $n = 167$, $p = 0.001$) (*Figure 12* *Figure 12*).

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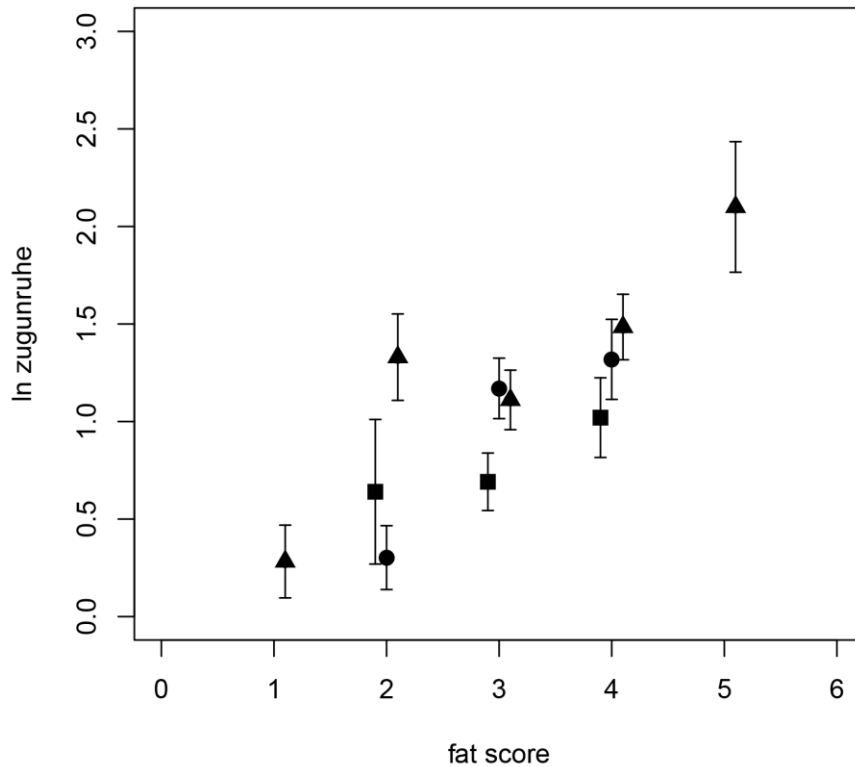


Figure 12 Log-transformed intensity of Zugunruhe (mean \pm SE) plotted against fat score for black redstart (circles), European robin (triangles) and European stonechat (squares). Zugunruhe was significantly correlated with fat score, with no specific effect of the species. See text for statistical details

By applying a Quade's Test, a non-parametric analysis of covariance, we tested if the correlation found in this global analysis was influenced by the factor species. The analysis showed no significant effect of the species on the relationship between Zugunruhe and fat ($F_{2,157} = 2,673$, $p = 0,072$).

For each species, we extracted a single PCA component from the variables fat score, muscle score and body mass, termed CONDITION. In black redstarts, CONDITION (eigenvalue 1.600) explained 53.0 per cent of the total variance, and its correlations with each variable were as follows: body mass, 0.890; fat, 0.900; and muscle, -0.030. In robins, CONDITION (eigenvalue 1.950) explained 65.0 per cent of the variance, and its correlations were as follows: body mass, 0.860; fat, 0.830; and muscle, 0.720. In stonechats, CONDITION (eigenvalue 1.690) explained 56.0 per cent of the variance, and its correlations were as follows: body mass, 0.920; fat 0.920; and muscle, 0.010. In black redstarts, CONDITION was significantly positively correlated with the amount of Zugunruhe; in particular, CONDITION was significantly positively correlated with Zugunruhe during the first part of the night, and positively, but not significantly, correlated with Zugunruhe during the second part of the night (*Table 1; Figure 13a, 13b, 13c*). In robins, CONDITION was significantly positively correlated with the amount of Zugunruhe; specifically, CONDITION was significantly positively correlated with Zugunruhe both during the first and the second part of the night (*Table 1; Figure 13d, 13e, 13f*). Finally in stonechats, the overall correlation between CONDITION and Zugunruhe was not significant; however, CONDITION was significantly positively correlated with Zugunruhe during the first part of the night, but not with Zugunruhe during the second part of the night (*Table 1; Figure 13g, 13h, 13i*). The amount of diurnal activity was not significantly correlated with CONDITION in all species (Table 1).

Statistics (r_p)	Black redstart (51)		European robin (98)		European stonechat (17)	
	R	<i>P</i>	R	<i>P</i>	R	<i>P</i>
<i>Zugunruhe</i>	0.325	0.021	0.336	0.001	0.349	0.169
<i>Zugunruhe during the first part of the night</i>	0.297	0.036	0.349	0.0004	0.556	0.021
<i>Zugunruhe during the second part of the night</i>	0.255	0.073	0.253	0.015	-0.111	0.670
<i>Diurnal activity</i>	0.118	0.414	-0.167	0.100	-0.202	0.436

Table 1 Correlations (Pearson) between Zugunruhe, Zugunruhe during the first part of the night, Zugunruhe during the second part of the night, and Diurnal activity and the factor CONDITION extracted from the variables body mass, fat score and muscle score by means of Principal Component Analysis. Bold typeface indicates statistically significant correlations

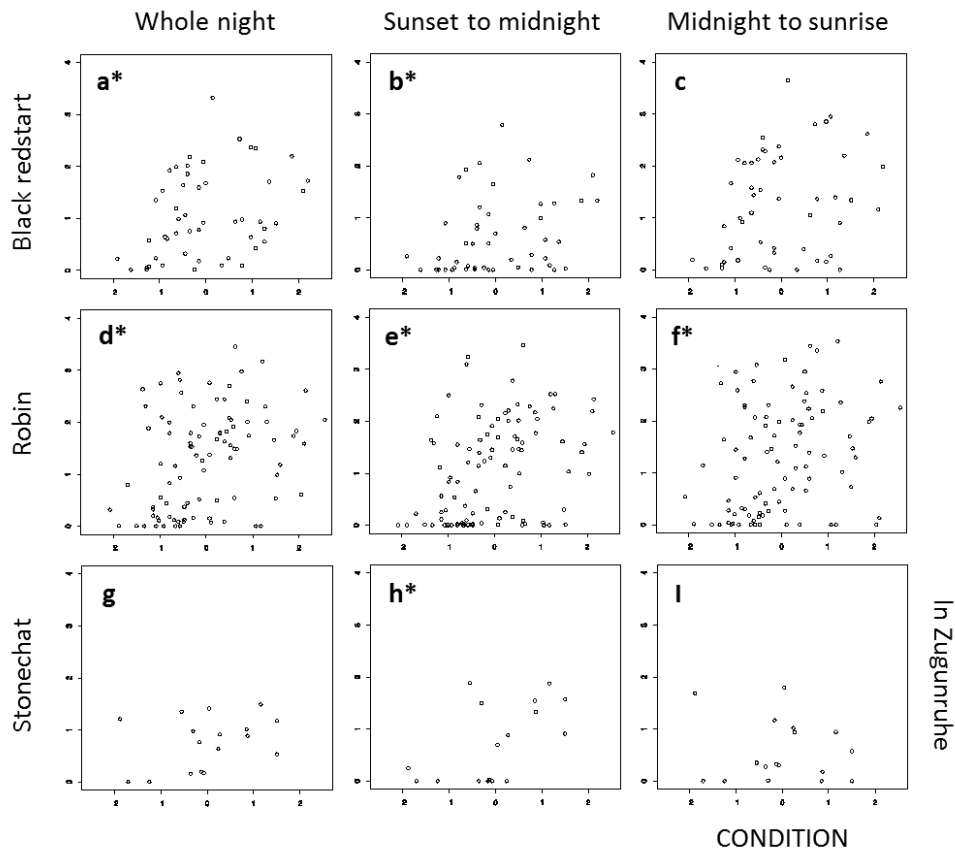


Figure 13 Scatterplots of log-transformed activity variables against the principal component CONDITION extracted from muscle score, fat score and body mass. For each species (top: black redstart, a, b, c; middle: European robin, d, e, f; bottom, European stonechat, g, h, i) we show Zugunruhe across the whole night (left, a, d, g), from sunset to midnight (middle, b, c, h) and midnight to sunrise (right, c, f, i). The stars (*) beside the panel letter indicates a significant correlation. See Table 1 for the statistics

Discussion

Our results show that in black redstarts and European robins caught at a spring stopover site body condition correlated with the amount of Zugunruhe. In stonechats, the correlation between body condition and Zugunruhe was significant only for the first part of the night. In all species, Zugunruhe was more strongly correlated with body condition in the first part of the night, and the lack of correlation with Zugunruhe across the whole night in stonechats was probably due to the smaller sample size. Overall, our study confirms that condition is a good indicator of migratory tendency in these three short-distance migrants.

The results support the hypothesis that energy reserves carried by birds when arriving at stopover sites are good predictors of stopover duration (Bairlein 1985; Biebach 1985), an idea that we confirmed in previous studies on long-distance migrants (Fusani et al. 2009; Goymann et al. 2010). Thus, energy reserves appear to be one of the main determinants of stopover duration, independently of the strategy of migration. Since we used an identical experimental approach and setup in the present study on short-distance migrants and in the previous study on long-distance migrants (Fusani et al. 2009), we are confident that the relationship between body condition and the amount of Zugunruhe reveals actual similarities in the control of migratory programmes between short- and long-distance migrants. One interesting difference is the possible contribution of the muscle to overall condition: differently from what observed in three species of long-distance migrants (Fusani et al. 2009), in black redstarts and stonechats the factor CONDITION was not significantly accounted by the muscle score.

Our study shows that individuals of the three study species express more intense Zugunruhe in the first part of the night. Telemetric data on European robins have shown that the take-off time is shifted towards the beginning of the night in birds having higher fuel loads and during shorter nights (spring versus autumn), suggesting that departure time is dependent on fuel loads and on night duration (Bolshakov et al. 2007). Moreover, data on caged birds have shown that migration is stimulated by darkness (Gwinner 1967) and reported a quiescent aphasic phase during sunset, in preparation to the migration (Ramenofsky et al. 2003). In this study, we used individuals with fat score 2 or more, excluding thus very lean birds, and we conducted the fieldwork during spring migration. Our results are in agreement with these observations and confirm that individuals having a sufficient amount of fuel load preferably perform their migratory flights in the first part of the night during spring migration.

Several external factors, such as weather condition and food availability, and internal factors, such as endogenous programmes, interact and thus influence the migratory programme and the decision of staying at a stopover site or resuming migration (Jenny & Shaub 2003). Overall, spring migration is thought to depend on a time-minimization strategy (Hedenström 2008), because birds arriving earlier at the breeding grounds may obtain better territories and mate earlier, thus enhancing their reproductive success compared to birds arriving late (Kokko 1999). The results of the present study, however, suggest that migratory behaviour strongly depends on the body condition of the animals and the time-minimization strategy should not overcome the necessity for short-distance migrants in poor condition to regain energy reserves. Endogenous circannual mechanisms controlling migratory parameters are particularly well developed in long-distance migrants, leading to a high temporal precision (Bairlein & Gwinner 1994; Helm et al. 2005). In fact, the timing of migration in long-distance migrants probably mainly relies on endogenous rhythms and photoperiod cues (Both & Visser 2001; Coppack & Both 2002). On the other hand, migratory schedules in short-distance migrants, which are characterized by shorter migration routes between wintering and breeding grounds, may be more flexible and the total duration of migration may depend more on the physiological condition and environmental factors. According to this hypothesis, our findings show that migratory behavior of short-distance migrants is finely modulated by energy condition. Moreover, we confirmed previous observations revealing that lean birds are less active at night than fat birds just after the overcoming of the ecological barrier (Yong & Moore 1993).

A novel contribution of our study was the capacity of our approach of separating the influence of environmental factors from that of internal factors. Our study birds were kept indoor in cages and in similar conditions during the recordings. Therefore, their behaviour was not affected by factors such as wind or cloud cover. The capture and the experiments, moreover, were conducted across the entire migratory season, thus calendar day and weather conditions preceding capture had no systematic effects on the results. Although it is likely that these factors introduced variability in the sample, the large sample size allowed clear relationships between condition and migratory behaviour to be uncovered.

Eikenaar & Bairlein (2013) demonstrated that birds maintained in captivity under fasting conditions increase *Zugunruhe*, showing that the motivation to migrate, quantified by *Zugunruhe*, is affected by changes in food availability. This result is in line with observational field studies showing that migrants are more likely to depart from a stopover

site when food is scarce than when food is plentiful (Bibby & Green 1981; Ottich & Dierschke 2003). Experiments suggest that stopover duration is very short if fuel deposition rate is low or negative (Biebach 1985; Gwinner et al. 1985; Yong & Moore 1993). Previous studies on three species of trans-Saharan migratory passerines found that all birds departed in the evening of the arrival day at sites with low food availability, whereas at sites with high food availability lean birds stayed at least one day (Biebach et al. 1986). Preliminary data from our research station on Ponza suggest that this stopover site probably provides opportunities to regain fat reserves (Trez et al. unpublished). Hence, it is likely that stopover duration is not influenced by the lack of food availability and that lean birds prolong stopover to replenish energy reserves.

A few studies had previously demonstrated the role of body condition on stopover duration in short-distance migrants, although the influence of environmental factors could not be excluded. In black redstarts, stopover duration is longer in individuals with low fat stores, and birds generally resume migration when weather conditions are favourable (Morganti et al. 2011). Moreover, in European robins departure decision depends on simultaneous action of both energetic and weather factors (Bulyuk & Tsvey 2013).

In all three species, day activity was not significantly correlated with body condition. These results contrast with previous reports of higher diurnal activity of lean birds at a stopover site, observed in the long-distance migrant, the garden warbler (Bairlein 1985), and in four species of North American thrushes that cross the Gulf of Mexico to reach their wintering areas (Yong & Moore 1993), which could reflect the simultaneous needs of foraging and minimizing the exposure to predators. In robins making stopovers on the Courish Spit, the proportion of recaptured individuals is nearly 4-fold lower in fat birds than in lean individuals (Titov 1999), suggesting a diurnal pattern of activity in lean birds. One possible explanation for the discrepancy between our results and previous work could lie in the pattern of migration and the frequency and distribution of the stopovers, leading to different strategies of fat accumulation through the migratory flights and different decisions during stopovers. Hence, diurnal activity is supposed to depend on the simultaneous effect of numerous factors, not only body condition, food availability and predation risk, but also the need to replenish energy reserves in relation to the stopover position along the migratory route.

In conclusion, our study revealed the influence of physiological condition on stopover duration in short-distance migrants. This adds evidence regarding the importance of fuel

resources in determining the decision of birds to resume migration or prolong stopover, and confirms that short- and long-distance migrants follow similar stopover strategies after crossing an ecological barrier.

Chapter 2: food intake and stopover decision

Introduction

Every year millions of small passerine birds migrate from Northern Europe to sub-Saharan Africa and back, covering large distances and facing substantial ecological barriers such as seas and deserts. Most birds do not complete migration in a single flight, but engage in several stopovers to accumulate energy resources, recover from fatigue, or wait for favorable weather conditions (Carmi et al. 1992; Klaassen 1996; Schmaljohann et al. 2007). Migratory birds are under time pressure to reach both their breeding grounds in spring (Kokko 1999) and their wintering grounds in autumn (Delingat et al. 2006), and hence they are expected to optimize migration pace. Most of the migration period is spent at stopover sites (normally more than 80%) (Hedenström & Alerstam 1997), and thus the number and duration of stopovers determines the overall pace of migration. Optimal migration theory predicts that stopover time should be reduced when birds experience high fuel deposition rates (Alerstam & Hedenström 1998), and they should then leave a stopover site as they reach the appropriate optimal fuel load (Alerstam & Lindström 1990). Therefore, birds arriving at a stopover site in poor condition should require longer stopovers or should be under stronger pressure to increase their fuel deposition rate to keep their stopover as short as possible. Two main optimal strategies have been proposed: time minimization, where migrants reduce the total time spent for migration to a minimum; and energy minimization, where migrants reduce the total amount of energy spent on migration to a minimum (Alerstam & Lindström 1990).

The dependency of stopover duration on individual condition has not been consistently shown in field studies that assessed the proportion of migrants that spent more than one day at a stopover site. Condition-dependence was shown in several studies conducted in autumn (Arizaga et al. 2008; Bairlein 1987, 1992; Biebach et al. 1986; Biebach 1985; Cherry 1982; Morris 1996; Smolinsky et al. 2013) with some exceptions (Schmaljohann et al. 2013; Tsvey et al. 2007). One study found that in long-distance migrants stopover duration depended mostly on condition, whereas in short-distance migrants stop-over duration did not depend on condition (Bairlein 1985). Another study found condition-dependence only at one of three sites in three species studied during autumn migration (Schaub et al. 2008). During spring migration, the patterns are more variable depending on species and location. Many studies showed condition-dependent stopover durations (Cherry 1982; Fusani et al. 2009; Gannes 2002; Goymann et al. 2010; Kuenzi et al. 1991;

Loria & Moore 1990; Lupi et al. 2016; Moore & Kerlinger 1987; Tenan & Spina 2010; Yong & Moore 1993) while others did not (Maggini & Bairlein 2011; Morris 1996; Safrieli & Lavee 1988; Salewski & Schaub 2007). The large number of studies with conflicting results implies that condition-dependency of stopover duration may vary among species, migratory season, and location. Most of these studies, though, were observational and based on recaptures of banded birds. The few studies measuring patterns of departure from stopover under more controlled laboratory conditions consistently showed that lean birds showed less nocturnal activity than fatter birds (Eikenaar & Schläfke 2013; Fusani et al. 2009; Lupi et al. 2016). By keeping birds in cages overnight, these studies took advantage of the fact that the disposition to migrate is reflected by the intensity of nocturnal activity (or migratory restlessness, hereafter *Zugunruhe*) in caged birds (Berthold et al. 1972; Eikenaar et al. 2014). However, the above-mentioned studies either did not provide food and thus refueling opportunities to the birds (Lupi et al. 2016) or did not measure food intake (Eikenaar & Schläfke 2013; Fusani et al. 2009). Recently it has been shown that fuel loss during fasting rather than initial condition is a better predictor for *Zugunruhe* in birds caged during migration (Eikenaar & Bairlein 2014). These data indicate that stop-over decisions might differ depending on whether birds were given the opportunity to refuel during the day or not. Particularly in spring, when birds are under strong time pressure to reach their breeding grounds (Bêty et al. 2003; Cooper et al. 2011; Currie et al. 2000; Descamps et al. 2011; Gunnarsson et al. 2006; Kokko 1999; Lozano et al. 1996; Reudink et al. 2009; Smith & Moore 2005), lean birds should resume migration earlier if they are able to refuel efficiently at a stopover site. However, there might be limitations to immediate refueling after landing, since many birds reduce their digestive tract to save energy for their flight (Hume & Biebach 1996; Piersma 1998). Therefore, readily available food does not necessarily translate into immediate successful refueling.

Here, we tested whether garden warblers (*Sylvia borin*) and European robins (*Erithacus rubecula*, hereafter robins) behaved as time- or energy minimizers when stopping over on the island of Ponza (central Italy) after a long-distance sea crossing during spring migration. The two species differ strongly in migration patterns. Garden warblers are long-distance migrants and the populations that come through Ponza need to fly over the Sahara Desert and the Mediterranean Sea before they finally reach their breeding grounds in Northern Europe. In contrast, robins are short-distance migrants and the populations that come through Ponza migrate from Northern Africa to Italy or Central Europe (Spina & Volponi 2008). It has been observed that both species show similar patterns of condition-

dependent stopover behaviour in Ponza, i.e. lean birds stay longer than fat birds (Fusani et al. 2009; Goymann et al. 2010; Lupi et al. 2016). We captured birds from both species and held them temporarily in captivity. In contrast to previous studies, we offered the birds refueling opportunities and monitored food consumption and body mass change to obtain a measure of their fuel deposition rate. At the same time, we measured diurnal activity and Zugunruhe to assess migratory disposition. If birds behaved like time-minimizers, we expected them to resume migration immediately after refueling, and their propensity to migrate should increase with increasing refueling. If they behaved as energy-minimizers departure should be condition-dependent, but should not depend on food intake.

Material and methods

The study was carried out between 19 March and 19 May 2012 at the Ponza Research Station (www.inanellamentoponza.it), located on the island of Ponza (40°50' N, 12°58' E; 9.87 km²), about 50 km from the Tyrrhenian coast of Italy. Garden warblers and robins were trapped with mist nets, which were continuously monitored between sunrise and sunset. Upon capture they were ringed and their body condition was assessed through scoring of fat and pectoral muscle (*Figure 14*). Fat was scored on a scale from 0 (no visible fat) to 8 (fat completely covering the pectoral muscle) and pectoral muscle was scored on a scale from 0 (muscle concave) to 3 (muscle bulging, sternum no longer visible) following Bairlein (1994).



Figure 14 Scoring of fat and pectoral muscle

We used 63 garden warblers and 88 robins for this experiment. To let the birds accustom to the housing conditions, only birds that were captured in the morning hours (before 13:00) were used. After ringing, all individuals were weighed and immediately transferred to individual custom-built fabric cages (50 x 30 x 30 cm) inside a room close to the capture site. These cages have been used in a number of previous studies (Bauchinger et al. 2008; Fusani et al. 2009; Fusani et al. 2011; Fusani et al. 2013; Lupi et al. 2016) and have been optimized for short-term housing of migratory passerines. In every cage an infrared sensor attached to the wall detected each bird's movement and a custom-made software summarized the movement counts in 2-minute intervals (*Figure 15*). Depending on capture success, up to 24 birds per day were tested simultaneously.



Figure 15 Custom-built fabric cages and recording equipment on the floor

The birds were provided with water ad libitum and with 8 ± 1 g of a mixture of dry insect food (Aleckwa, Germany), boiled egg, and banana, and were exposed to a natural photoperiod. The food was weighed before the birds were placed into the cage, and the remaining food (including what had been scattered around the cage by the birds) was weighed after releasing the birds in the next morning. To account for evaporative loss of food weight, a reference bowl of food was placed into an empty cage and weighed at the

same times. Food intake was then calculated by subtracting the weight of the food bowl at the end from the weight of the bowl at the beginning of the experiment, and corrected for evaporation using the difference in weight of the control food bowl.

Zugunruhe during the first part of the night was calculated as the mean number of movements per 2-minutes interval between civil twilight (Central European Time) and midnight (00:00). We decided to interrupt the measurements at this time because some of the birds were sampled for blood after midnight for another experiment. However, departure from a stopover site is likely to happen during the first part of the night in the majority of nocturnal migrants (Bolshakov et al. 2007; Bruderer 1997; Goymann et al. 2010; Gwinner 1967; Ramenofsky et al. 2003). Diurnal activity was calculated as the mean number of movements per 2-minutes interval between 13:00 and civil twilight. Before releasing them in the morning at 06:00, all birds were weighed again.

A total of 87 birds (38 garden warblers and 49 robins) were equipped with a logger (1.5 g) for a separate experiment not reported here. We accounted for possible effects of the logger on locomotor activity in the statistical analysis. In addition, locomotor activity data were excluded from the analysis during the period in which a researcher was present in the room until one hour after the end of the disturbance, to remove possible effects of this disturbance from the results.

We extracted a body condition index called *CONDITION* by conducting a principal component analysis (PCA) on body mass, fat score and muscle score at capture (as in Fusani et al. 2009; 2011). In garden warblers, *CONDITION* (eigenvalue 2.34) explained 78 % of the total variance, and the individual variable loadings were as follows: body mass 0.90; fat 0.89; and muscle 0.86. In robins, *CONDITION* (eigenvalue 1.76) explained 59 % of the total variance, and the individual variable loadings were as follows: body mass 0.88; fat 0.88; and muscle 0.47. We performed linear regression models to test for the effect of *CONDITION*, food intake, logger and the interaction between *CONDITION* and food intake on Zugunruhe and diurnal activity, separately for garden warblers and robins. We calculated variance inflation factors (VIFs) for all models to control for collinearity of variables. VIFs were never higher than 2. These models were compared to GAMs performed using the same explanatory variables in order to identify possible non-linear relationships. The results of the GAMs were suggesting that linear relationships were a good approximation of the data. Adjusted R² values were comparable to the linear regression models and AIC of the GAMs were always higher than those of the linear

regression models. For this reason, we are only presenting the results of the linear regression models here. We performed Student's t-tests to check for statistical differences in food consumption between garden warblers and robins. Because the variance in food consumption was highest at intermediate fat scores (fat score 1, 2, and 3, see results **Figure 16**) in both species, the linear regression models were run on two different datasets for each species: 1) the full dataset, including all individuals, 2) an intermediate fat scores dataset, including only the individuals with fat scores 1 to 3. The models were checked for homogeneity of residuals and heteroscedasticity by plotting residuals (Crawley 2007). All statistical analyses were performed with R 3.0.2 (R Core Team 2014). Due to missing values for food intake, a total of 62 of 63 garden warblers and 85 of 88 robins were included in the linear models with the full dataset, and 51 of 52 garden warblers and 75 of 80 robins were included in the linear models with the intermediate fat scores dataset.

Results

Multiple regression models showed that food intake affected Zugunruhe and diurnal activity in garden warblers: higher food intake was associated with higher intensity of Zugunruhe when considering the intermediate fat scores dataset and with lower diurnal activity in both datasets (**Table 2, 3**). Garden warblers with loggers showed a lower intensity of Zugunruhe than birds without loggers (**Table 2**). Birds with higher CONDITION values showed more Zugunruhe and less diurnal activity in both datasets (**Table 2, 3; Figure 17a, 17c**). In robins, food intake did not affect Zugunruhe or diurnal activity (**Table 2, 3**). When considering the full dataset, birds with higher CONDITION values showed more Zugunruhe (**Figure 17b, 17d**). High CONDITION values were correlated with lower diurnal activity when considering the intermediate fat scores dataset (**Table 3**). In two cases (full dataset: diurnal activity; intermediate fat scores dataset: Zugunruhe) the variance explained by the models for the robin was very low and the models were not significant.

Overall, garden warblers ate significantly more (mean \pm 95% CI: 1.686 ± 0.233 g) than robins (mean \pm 95% CI: 0.634 ± 0.107 g; t-test: $t = 4.102$, $p < 0.001$; **Figure 16**), even though the two species had a similar body size (garden warbler: 16.8 ± 1.5 g; robin: 15.7 ± 1.2 g). CONDITION was negatively correlated with food intake in both garden warblers (Pearson's correlation: $r = -0.398$, $p = 0.002$) and robins ($r = -0.299$, $p = 0.006$).

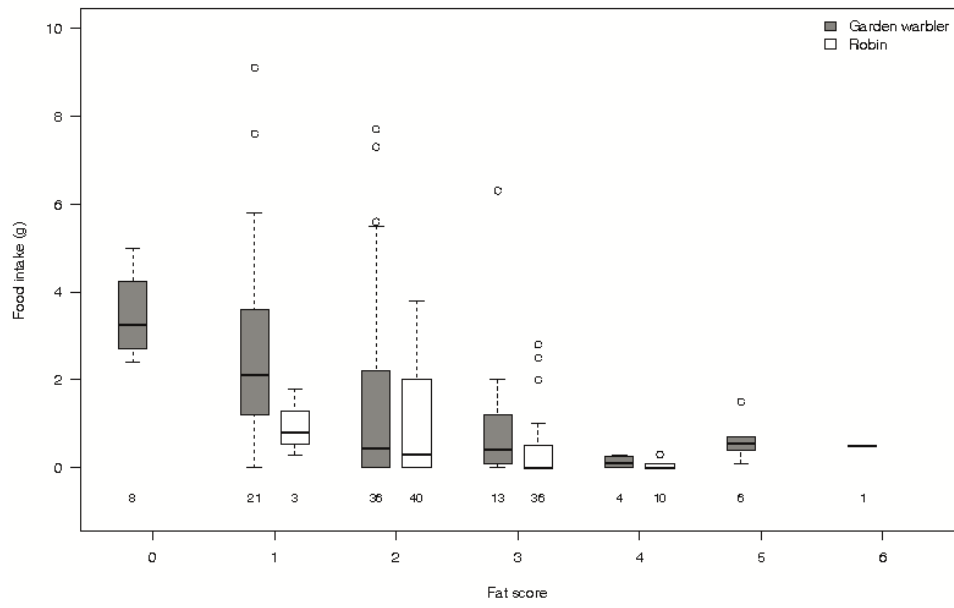


Figure 16 Food intake by fat score in garden warblers (grey) and robins (white). Sample sizes are indicated below the boxes

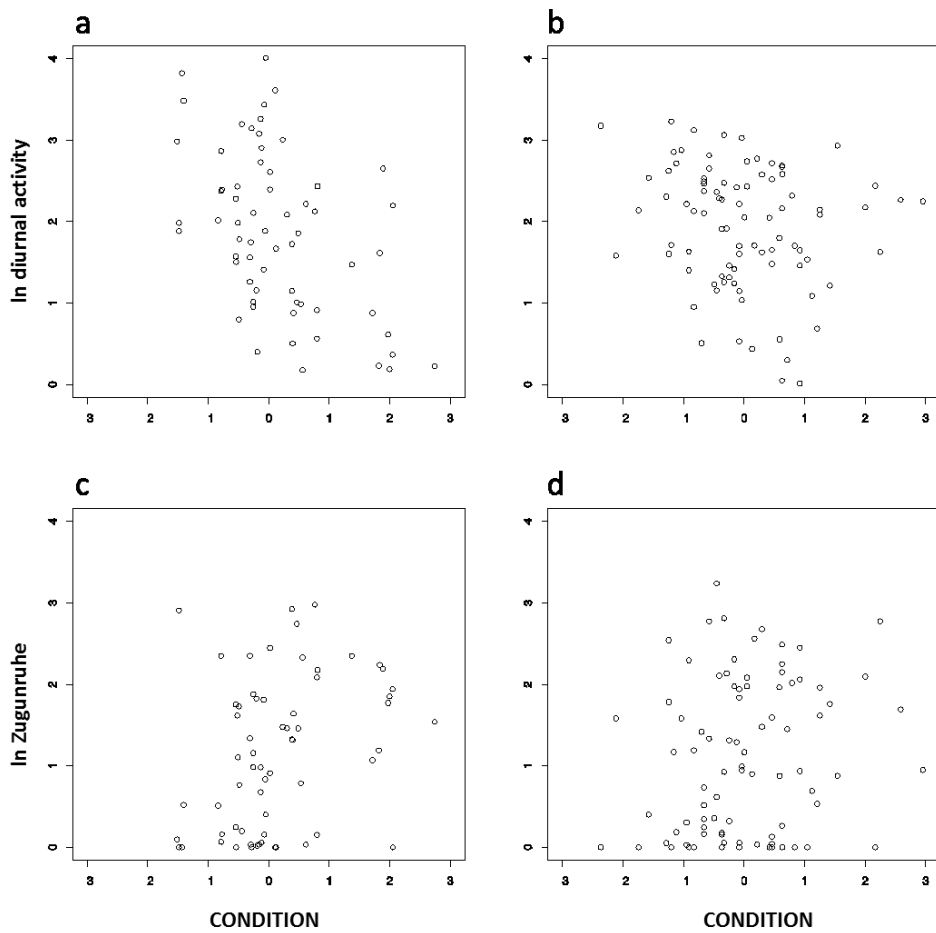


Figure 17 Relationships between CONDITION and diurnal activity or Zugunruhe in garden warblers (a, c), and robins (b, d). There was a significant negative correlation between CONDITION and diurnal activity ($r_p = -0.451$, $p = 0.000$, a) and a positive correlation between CONDITION and Zugunruhe ($r_p = 0.442$, $p = 0.000$, c) in garden warblers. In robins, CONDITION was significantly positively correlated with Zugunruhe ($r_p = 0.246$, $p = 0.019$, d), but not with diurnal activity ($r_p = -0.167$, $p = 0.115$, b)

FULL DATA SET

Zugunruhe		Effect size	Std. Error	t value	Pr(> t)
Garden warbler	(Intercept)	1.136	0.190	5.968	0.000
	CONDITION	0.394	0.140	2.817	0.007
	food intake	0.187	0.114	1.644	0.106
	Logger	-0.423	0.209	-2.026	0.047
	CONDITION: food intake	0.111	0.153	0.723	0.472
Robin	(Intercept)	1.099	0.162	6.771	0.000
	CONDITION	0.294	0.116	2.528	0.013
	food intake	0.232	0.127	1.822	0.072
	Logger	-0.231	0.203	-1.136	0.259
	CONDITION: food intake	0.043	0.138	0.312	0.756
Diurnal activity					
Garden warbler	(Intercept)	2.183	0.202	10.801	0.000
	CONDITION	-0.530	0.149	-3.563	0.001
	food intake	-0.292	0.121	-2.418	0.019
	Logger	0.068	0.222	0.309	0.759
	CONDITION: food intake	-0.208	0.162	-1.282	0.205
Robin	(Intercept)	2.128	0.127	16.758	0.000
	CONDITION	-0.168	0.091	-1.854	0.068
	food intake	-0.053	0.100	-0.529	0.598
	Logger	-0.299	0.159	-1.881	0.064
	CONDITION: food intake	0.029	0.108	0.265	0.792

Table 2 Outcome of the multiple regression models for garden warbler and robin using the full data set. The dependent variables were Zugunruhe and diurnal activity. Bold typeface represents significant effects. Statistics of the models with Zugunruhe as dependent variable: garden warbler adj. $R^2 = 0.205$, $F_{4,57} = 4.939$, P of the model = 0.002; robin adj. $R^2 = 0.0699$, $F_{4,80} = 2.578$, P of the model = 0.044. Statistics of the models with diurnal activity as depend variable: garden warbler adj. $R^2 = 0.279$, $F_{4,57} = 6.908$, P of the model = 0.000; robin adj. $R^2 = 0.047$, $F_{4,80} = 2.024$, P of the model = 0.099

INTERMEDIATE FAT SCORES DATA SET

Zugunruhe		Effect size	Std. Error	t value	Pr(> t)
Garden warbler	(Intercept)	0.995	0.185	5.376	0.000
	CONDITION	0.449	0.136	3.301	0.002
	food intake	0.214	0.094	2.292	0.027
	Logger	-0.311	0.211	-1.472	0.148
	CONDITION: food intake	0.126	0.118	1.071	0.290
Robin	(Intercept)	1.048	0.180	5.835	0.000
	CONDITION	0.209	0.136	1.537	0.129
	food intake	0.229	0.118	1.933	0.057
	Logger	-0.253	0.221	-1.144	0.256
	CONDITION: food intake	0.055	0.123	0.448	0.656
Diurnal activity					
Garden warbler	(Intercept)	2.353	0.220	10.716	0.000
	CONDITION	-0.607	0.161	-3.762	0.000
	food intake	-0.214	0.111	-1.932	0.060
	Logger	-0.094	0.250	-0.376	0.708
	CONDITION: food intake	-0.055	0.140	-0.392	0.697
Robin	(Intercept)	2.148	0.140	15.315	0.000
	CONDITION	-0.299	0.106	-2.817	0.006
	food intake	-0.043	0.092	-0.462	0.646
	Logger	-0.336	0.172	-1.947	0.056
	CONDITION: food intake	0.094	0.096	0.976	0.333

Table 3 Outcome of the multiple regression models for garden warbler and robin using the intermediate fat scores dataset. The dependent variables were Zugunruhe and diurnal activity. Bold typeface represents significant effects. Statistics of the models with Zugunruhe as dependent variable: garden warbler adj. $R^2 = 0.294$, $F_{4,46} = 6.213$, P of the model = 0.000; robin adj. $R^2 = 0.041$, $F_{4,70} = 1.798$, P of the model = 0.139. Statistics of the models with diurnal activity as depend variable: garden warbler adj. $R^2 = 0.247$, $F_{4,46} = 5.088$, P of the model = 0.002; robin adj. $R^2 = 0.112$, $F_{4,70} = 3.323$, P of the model = 0.015

Discussion

Optimal migration theory predicts that birds should depart from a stopover site with specific energy stores to optimize their flight range (Alerstam & Lindström 1990). The optimal fuel load required to leave the stopover site is predicted to be dependent on the fuel deposition rate in time-minimizing migrants, while energy-minimizers should maintain a constant optimal fuel load irrespective of fuel deposition rate (Alerstam & Hedenström 1998). This study confirms previous findings that birds with sufficient energy stores are more likely to leave the stopover site than birds with little energy stores (Eikenaar & Schläfke 2013; Fusani et al. 2009; Goymann et al. 2010; Lupi et al. 2016). The latter studies also observed that the higher Zugunruhe in fatter birds was coupled with a lower activity during the day. The large individual variability in food intake in intermediate fat classes prompted us to investigate how fuel deposition rate affected departure decisions (as predicted by Zugunruhe) (Eikenaar et al. 2014) of such birds. The results indicated different patterns between the two species. In garden warblers, higher food intake resulted in more Zugunruhe, as expected in a species that minimizes migration time. In contrast, food intake did not affect Zugunruhe in robins, which thus followed the pattern predicted by energy-minimization.

Time minimization is consistently found, especially during spring migration and in long-distance migrants (Dierschke et al. 2005; Farmer & Wiens 1999; Karlsson et al. 2012; Lindstrom & Alerstam 1992; Maggini & Bairlein 2010; Scheiffarth et al. 2002). Garden warblers, as strict long-distance migrants, fit well in this scenario. The strong endogenous regulation of migration in this species (Gwinner & Wiltschko 1978; Gwinner 1996) causes a very consistent response to intrinsic and extrinsic factors (like e.g. body condition and food availability) which is not masked by experimental conditions such as temporary captivity. On the contrary, food intake was rather low for all fat classes in robins. This would be expected if birds have a reduced digestive tract as often observed in migrants after long-distance flights, such as when they are crossing the Mediterranean Sea (Bauchinger et al. 2005; Hume & Biebach 1996; Karasov et al. 2004; McWilliams & Karasov 2005). Alternatively, a low fuel deposition rate could reflect capture stress (Carr 2002). In robins that did refuel, though, food intake had no effect on Zugunruhe, mirroring the expected pattern for energy-minimizers. Because robins are short-distance migrants, they might follow a different strategy. A study by Dierschke and Delingat (2001) showed that northern wheatears (*Oenanthe oenanthe*) left a stopover site close to their breeding area quickly and independently from refuelling rate and weather conditions. In contrast

birds that faced a large ecological barrier finely adjusted their departure according to body condition. Thus, robins in our study might have shown the observed patterns in food intake and Zugunruhe because they were close to their breeding grounds. Robins caught on Ponza are likely to breed in close locations on mainland Italy (Spina & Volponi 2008). It is also possible that time-minimizing birds switch to energy-minimizing when approaching the breeding grounds to save energy for the breeding season. For instance, arriving at the breeding grounds with sufficient energy stores has been shown to increase fitness in some migrants (Descamps et al. 2011; Sandberg & Moore 1996; Smith & Moore 2003).

It has already been shown that food availability affects Zugunruhe (Fusani et al. 2011). In this previous study, however, food intake was not measured. The present study shows that the actual food intake rather than the simple presence of food increases Zugunruhe, at least in garden warblers. Birds of comparable, intermediate condition that had access to the same amount of food showed different levels of Zugunruhe depending on whether they ate or not (with Zugunruhe increasing with increasing food intake). This observation indicates that there might be a large component of individual variability in food consumption that goes beyond body condition. Personality traits such as neophobia (when presented a new environment) (Mettker-Hofmann et al. 2009) and differential responsiveness to stressors, or endogenous stressors such as parasite burden (Klaassen et al. 2012; van Gils et al. 2007) could be partly responsible for the (large) unexplained variance in the response of Zugunruhe to condition and food intake.

Our results revealed that birds in intermediate condition showed the largest variability in their behavioural response to condition and food availability. Fuel deposition rate in such birds might be highly variable depending on external factors such as food availability, but also on intrinsic factors that may affect the propensity or the ability to refuel, such as physiological constraints (digestive tract reduction) or behavioural responses to stressors (e.g. predators). We suggest a possible species-specific pattern of departure decisions in response to fuel deposition rates, which probably relates to minimization of either time or energy, though the possible confounding effects of capture stress have to be taken into account when studying birds during short-term restraint.

Chapter 3: ghrelin and stopover decision

Introduction

Birds fuel their migratory flights primarily through fatty acid metabolism (McWilliams et al. 2004; Piersma et al. 2005). Research from our group has shown that the physiological condition of the bird at the stopover site is the strongest internal predictor of stopover duration (Fusani et al. 2009; Goymann et al. 2010; Lupi et al. 2016). The extent of fat reserves, the main form of energy storage in birds, is strongly correlated with the disposition to migrate in captivity (Zugunruhe) (Fusani et al. 2009; Lupi et al. 2016) and the actual stopover duration in nature (Goymann et al. 2010). We have also shown that food availability per se influences Zugunruhe, indicating that food consumption might also have a direct regulatory effect (Fusani et al. 2011; Lupi et al. submitted, see 'Chapter 2: food intake and stopover decision'). Hence, we decided to look at hormones that reflect the nutritional state and/or feeding behaviour, hypothesizing for them the role of trigger for the behavioural and metabolic switches between stopovers and migration.

In vertebrates, there is a complex network of orexic/anorexic hormones regulating food intake and appetite (Furuse et al. 2007; Valassi et al. 2008; Volkoff et al. 2005). Ghrelin belongs to this class of hormones and it has been recently discovered in birds as an endogenous ligand for the growth hormone (GH) secretagogues-receptor (GHS-R) (Kojima et al. 1999). It is mainly secreted by proventriculus, the glandular part of the stomach, even though it is widely expressed in many tissues, such as pancreas, brain, duodenum, spleen, hypothalamus and liver (Chen et al. 2007; Kaiya et al. 2002; Richards et al. 2006; Wada et al. 2003). Avian ghrelin was first isolated from chicken proventriculus and found to be a 26 amino acid peptide, compared to the 28-amino acids peptide found in mammals (Kaiya et al. 2002) (*Figure 18*). In both mammals and non-mammals, ghrelin is acylated by n-octanoic or n-decanoic acids at the third amino acid (usually Ser³) (Hosoda et al. 2006; Kaiya et al. 2008) through the action of O-acyltransferase enzyme, GOAT (Gutierrez et al. 2008; Yang et al. 2008). The acyl- modification of des-acyl ghrelin, a form lacking acyl-modification of ghrelin, occurs in ghrelin-producing cells. In birds, the acyl-modification takes place in the proventriculus (Kaiya et al. 2002) and it is essential for ghrelin binding with its cognate receptor, the growth hormone secretagogues-receptor (GHS-R). It has been reported that fatty acids, that are the substrate for ghrelin modification, originate from dietary lipids (Yamato et al. 2005). Once both ghrelin and des-acyl ghrelin are released from the proventriculus and circulate through the body, it is

known that ghrelin exhibits multiple biological actions through GHS-R. However, in mammals the majority (80-90%) of the hormone secreted into the blood is des-acyl ghrelin (Ariyasu et al. 2002; K Toshinai et al. 2001). In fact, ghrelin rapidly disappears from the circulation due to the binding to the GHS-R in the systemic tissues (Akamizu et al. 2005). Furthermore, few studies suggest that ghrelin is rapidly des-acylated in the plasma, showing a reduced half-life compared to des-acyl ghrelin (Hosoda et al. 2004; Satou et al. 2010). The precise mechanism for the des-acylation of ghrelin in circulation remains to be clarified. In birds, des-acyl ghrelin level in the proventriculus and blood is yet unknown, although its level is thought to be higher than that of acylated ghrelin. At the beginning, des-acyl ghrelin was considered to be an inactive form of ghrelin, but recent evidence indicates that it might have biological activities, including regulation of appetite, through a receptor distinct from GHS-R (Asakawa et al. 2005; Chen et al. 2005; Toshinai et al. 2006). However, central administration showed no effects of des-acyl ghrelin on feeding behaviour in neonal chicks (Tachibana et al. 2011).

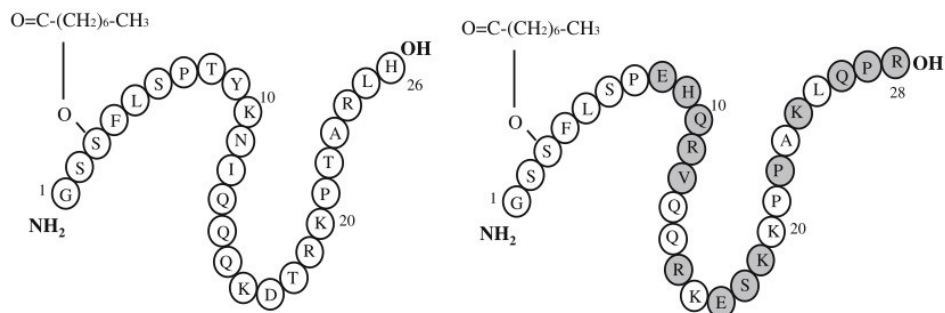


Figure 18 Chicken and human ghrelin sequences. Chicken ghrelin is a 26-amino acid peptide (left), whereas human ghrelin consists of 28-amino acids (right). Grey circles in human ghrelin indicate different amino acids compared to chicken ghrelin. From Kaiya et al. 2009

Although ghrelin is widely conserved among vertebrates, large differences have been observed in the study of its main functions. Ghrelin has growth hormone-releasing activity *in vivo* and *in vitro* in birds as in various mammalian species (Bhatti et al. 2002; Harrison et al. 2008; Hashizume et al. 2003; Hayashida et al. 2001; Kojima et al. 1999). In rodents, ghrelin is known to promote conservation of energy in lipid stores due to a stimulatory effect on adipose tissue accretion (Thompson et al. 2004), and enhance the catabolism of carbohydrate-rich fuels (Tschöp et al. 2000), leading to an increase in respiratory quotient

(RQ). In contrast, peripheral chicken ghrelin administration significantly reduced RQ for up to 14 h in ad libitum fed broiler chicks (Geelissen et al. 2006). A decrease of RQ suggests that ghrelin induces a reduction in lipogenic activity. This hypothesis was confirmed in a study showing that in one-day-old male broiler chicks peripheral administration of ghrelin downregulates the mRNA levels of the lipogenic enzyme fatty acid synthase (FAS), and two transcription factors (SREBP-1 and PPAR α) in liver, which is the major site of lipogenesis in avian species (Buyse et al. 2009). In mammals, ghrelin stimulates food intake (Higgins et al. 2007; Wren et al. 2001) through the orexigenic neuropeptide Y (NPY) and orexin. In contrast, ghrelin plays an inhibitory role in the regulation of food intake in quails and chickens when injected centrally (Chen et al. 2008; Furuse et al. 2001; Khan et al. 2006; Saito et al. 2002; Shousha et al. 2005). It is likely that central ghrelin may act through the corticotropin-releasing factor (CRF) system, which activates the hypothalamus-pituitary-adrenal (HPA) axis resulting in corticosterone (CORT) release from the adrenal glands. In fact, central injection of rat and chicken ghrelin inhibit food intake in neonatal chicks (Furuse et al. 2001; Saito et al. 2002a). Furthermore, ghrelin central injections increase stepping and vocalization, which are characteristic of hyperactive state in chick (Saito et al. 2002b), and central injections of CRF stimulate such agitated behaviour and inhibit food intake (Furuse et al. 1997a; Furuse et al. 1997b; Zhang et al. 2001).

In the present study, we investigated 1) the relationship between the extend of subcutaneous fat reserves and circulating ghrelin levels to assess its role on lipid metabolism, and 2) the central effects of peripheral administration of acyl- (hereafter 'ghrelin') and des-acyl ghrelin on food intake and migratory disposition (Zugunruhe). This is the first study investigating the role of ghrelin in a migratory bird, the garden warbler, with its unique metabolism and energy costs. In fact, all information obtained in previous studies refers to domesticated poultry. In chickens, ghrelin administration reduces the respiratory quotient and the lipogenesis in liver (Buyse et al. 2009; Geelissen et al. 2006). These observations suggest that ghrelin might function as a lipid sensor. Furthermore, ghrelin administration is known to inhibit food intake in quails and chickens. According these findings, we hypothesize that in our study species 1) increasing fat reserves are associated with higher levels of circulating ghrelin, and 2) ghrelin administration inhibits food intake and increases migratory disposition. A study found that central administration of des-acyl ghrelin did not show an effect on food intake in chicken (Tachibana et al. 2011). However, the overall knowledge of its physiological functions is scarce.

Furthermore, des-acyl ghrelin is thought to be a bioactive molecule and a product of des-acylation of circulating ghrelin, showing high concentration in blood and a longer half-life compared to ghrelin. Thus, we hypothesize that des-acyl ghrelin might induce central effects on food intake and migratory disposition.

Material and methods

We conduct our fieldwork study in Ponza. We used 317 garden warblers (*Sylvia borin*), caught with mist-nets during spring ringing operations from March to May. We collected physiological measurements, i.e. subcutaneous fat, thickness of pectoral muscle and body mass, plasma samples for ghrelin measurements and intensity of Zugunruhe, through individual cages for locomotor activity recording.

According with our two initial hypotheses, we conducted two experiments to test 1) the relationship between subcutaneous fat reserves and circulating ghrelin levels immediately after the capture, and 2) the effects of peripheral administration of ghrelin and des-acyl ghrelin on food intake and Zugunruhe, according to the extent of fat reserves.

Experiment 1 involved sampling at the nets during daytime, and collecting plasma samples within 3 minutes after capture in order to avoid a stress hormonal response (**Figure 19**). We measured the latency between capture and plasma collection and controlled for its effects on ghrelin levels in the statistical analyses. The collection of plasma sample for ghrelin measurement involved collecting around 200 μ l of blood from the wing vein with Na-Hep covered capillaries (**Figure 20**). We centrifuged samples immediately after collection, collected and measured the amount of plasma, normally around 50-70 μ l, with a Hamilton syringe. Then, we added 1M HCl at a ratio of 1/10 to the plasma to keep ghrelin in its acylated form. We finally stored the plasma sample in a freezer at -20°C within an hour.



Figure 19 Blood collection at the mist-nets



Figure 20 Collection of the blood with Na-Hep covered capillaries

Experiment 2 followed the 'overnight stay' procedure. Birds were caged by 12:00 with water ad libitum. We examined the effects of peripheral (intra-peritoneal, IP) injection of various recombinant chicken ghrelin and des-acyl ghrelin solutions on Zugunruhe (**Figure 21**). Recombinant chicken ghrelin and des-acyl ghrelin were purchased from Phoenix Pharmaceutical Inc. (CA, USA). Before the treatment, these peptides were dissolved in 0.9% NaCl saline and birds were randomly divided into experimental and control groups. By 13:00, four experimental groups were treated with different doses of ghrelin and des-acyl ghrelin, and a control group was treated with 0.9% NaCl saline. The solutions were administered using a micro syringe. We conducted 50 μ l IP injections of low dose of

ghrelin (L-GHR), high dose of ghrelin (H-GHR), low dose of des-acyl ghrelin (L-DGHR), high dose of des-acyl ghrelin (H-DGHR), and saline solution (SAL). The low and high doses of both the peptides were respectively of 2 and 10 $\mu\text{mol/l}$. Birds were given 3 g of food (mealworms) at the same time after the injection session (*Figure 22*). The following morning, we assessed the fat score, muscle score and body mass just before releasing birds and calculated the food intake by subtracting the final weight of the food bowl from the initial weight (3 g).



Figure 21 Preparation to the intraperitoneal (IP) injection



Figure 22 Food bowl (mealworms)

I conducted the hormonal measurement of plasma ghrelin (experiment 1) using radioimmunoassay procedure (RIA), in the laboratory of Dr. Hiroyuki Kaiya at the National Cerebral and Cardiovascular Institute, Osaka, Japan (*Figure 23*). The procedure involved the sample extraction by means of Sep-Pak Plus C18 cartridges; then, we conducted the radioimmunoassay using antibodies, primary antibody and anti-rabbit goat IgG serum, and labelled antigen, [Tyr29] - rat ghrelin, developed in the Kaiya Lab. We had validated the assay for our study species, reaching a sensitivity of 0.5 fmol/tube, which allows the determination of ghrelin concentration in individual samples.



Figure 23 Laboratory of Dr. Hiroyuki Kaiya, National Cerebral and Cardiovascular Institute, Osaka, Japan

In experiment 1, we centered latency values by calculating the mean of the entire vector, then scaling each element by subtracting the mean. Then, we transformed all the calculated values of ghrelin by taking the \log_{10} . Finally, we performed linear regression model to test for the effect of latency and fat score on ghrelin levels in garden warblers. We reduced the dataset by considering only individuals with fat score 0-3, because the number of individuals belonging to fat category 4 was statistically insufficient. Due to missing values, a total of 76 of 78 garden warblers were included in the linear model.

In experiment 2, we calculated Zugunruhe as the mean value of Zugunruhe during the first and the second part of the night, as we did in the previous studies (Chapter 1 and 2). We transformed all the calculated values of Zugunruhe and food intake by taking the log₁₀. We centered fat score values by calculating the mean of the entire vector, then scaling each element by subtracting the mean. Finally, we performed linear regression models to test for the effect of L-DGHR and H-DGHR treatments, and fat amount (hereafter 'fat') on Zugunruhe and food intake. This analysis was called 'DGHR-test' (des-acyl ghrelin test). The same analysis was performed to test the effect of L-GHR and H-GHR. The analysis was called 'GHR-test (ghrelin test)'. A total of 119 and 120 garden warblers were respectively included in the DGHR- and GHR-test.

Results

Fat score and ghrelin were positively correlated ($N = 78$, $r_S = 0.326$ $p = 0.004$, **Figure 24**) in experiment 1. Garden warblers with fat score 0, 1 and 2 showed differences in ghrelin level (**Figure 24**). In fact, according to Bayesian inference, if the credible interval of one group does not overlap with the mean of the other group then the difference is meaningful. Multiple regression models showed that both fat score and latency affect ghrelin in garden warbler: higher fat score were associated with higher ghrelin levels and higher latency was slightly associated with higher ghrelin levels (**Table 4**).

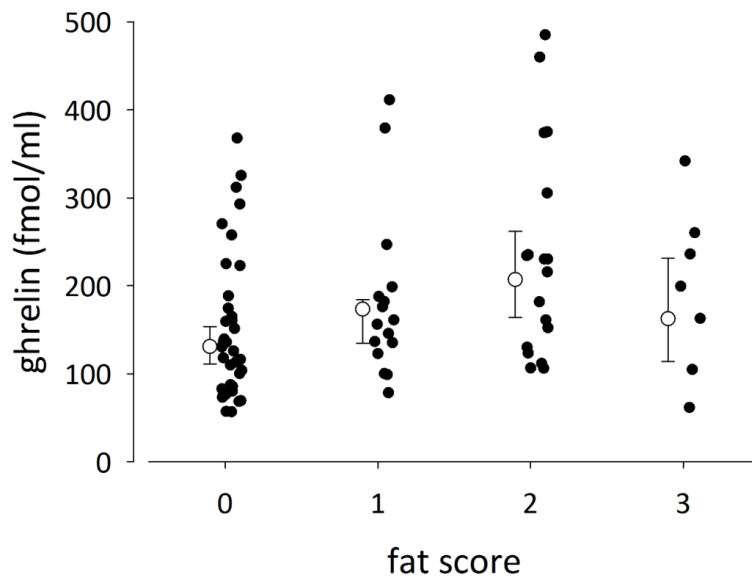


Figure 24 Mean ghrelin levels estimate and the lower and upper 95% credible interval for each fat score (0-3) in garden warbler (experiment 1)

Experiment 1: FAT SCORE AND LATENCY ON CIRCULATING GHRELIN

CIRCULATING GHRELIN	Effect size	Std. Error	t value	Pr(> t)	adj. R2	DF	F	P
(Intercept)	2.106	0.035	59.720	0.000				
Latency	0.064	0.025	2.592	0.012				
fat score 1	0.133	0.064	2.095	0.040	0.168	4,71	4.794	0.002
fat score 2	0.209	0.062	3.389	0.001				
fat score 3	0.105	0.083	1.265	0.210				

Table 4 Outcome of multiple regression models in garden warblers (experiment 1). The dependent variable was circulating ghrelin levels. Bold typeface represents significant effects

In experiment 2 relative to DGHR-test, multiple regression models showed that low dose des-acyl ghrelin treatment affects the intensity of Zugunruhe: low dose injected birds showed higher Zugunruhe than control birds (*Table 5; Figure 25*). Differently, high dose des-acyl ghrelin treatment did not affect Zugunruhe (*Table 5; Figure 25*). None of the treatments affected food intake (*Table 5*).

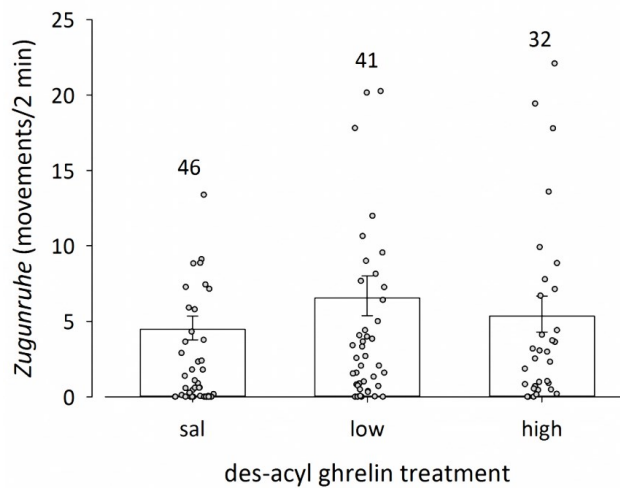


Figure 25 Mean Zugunruhe levels estimate and the lower and upper 95% credible interval for saline (sal), low dose (low), and high dose (high) of des-acyl ghrelin treatment in garden warblers (experiment 2, DGHR-test)

Experiment 2: DGHR-test								
ZUGUNRUHE	Effect size	Std. Error	t value	Pr(> t)	adj. R2	DF	F	P
(Intercept)	0.541	0.049	11.011	0.000				
high dose treatment	0.097	0.077	1.262	0.209	0.218	3,115	11.940	0.000
low dose treatment	0.202	0.071	2.833	0.005				
Fat	0.161	0.031	5.207	0.000				
FOOD INTAKE								
(Intercept)	0.347	0.021	16.448	0.000				
high dose treatment	-0.061	0.033	-1.845	0.068	0.295	3,115	17.490	0.000
low dose treatment	-0.043	0.031	-1.393	0.166				
Fat	-0.093	0.013	-6.968	0.000				

Table 5 Outcome of multiple regression models in garden warblers (experiment 2, DGHR-test). The dependent variables were Zugunruhe and food intake. Bold typeface represents significant effects

In experiment 2 relative to GHR-test, multiple regression models showed that high dose ghrelin treatment affects the intensity of Zugunruhe: high dose injected birds showed lower Zugunruhe than control birds (*Table 6; Figure 26*). However, the above-mentioned effect might hinge on a single data point showing the highest value of Zugunruhe in the SAL group. Differently, low dose ghrelin treatment did not affect Zugunruhe (*Table 6; Figure 26*). None of the treatments affected food intake (*Table 6*).

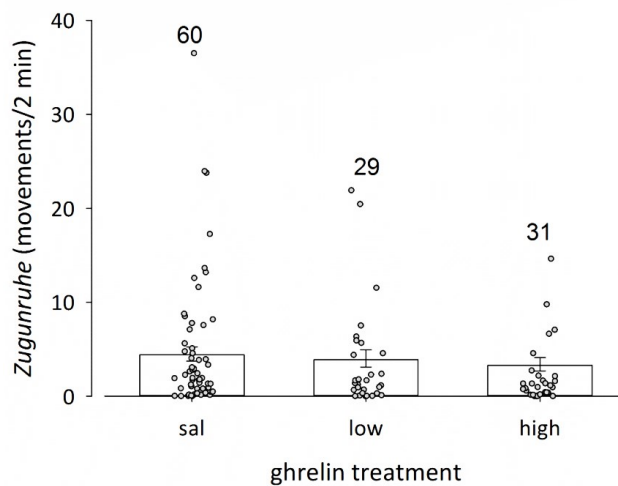


Figure 26 Mean Zugunruhe levels estimate and the lower and upper 95% credible interval for saline (sal), low dose (low), and high dose (high) of acyl ghrelin treatment in garden warblers (experiment 2, GHR-test)

Experiment 2: GHR-test

ZUGUNRUHE	Effect size	Std. Error	t value	Pr(> t)	adj. R2	DF	F	P
(Intercept)	0.530	0.048	11.079	0.000				
high dose treatment	-0.174	0.082	-2.111	0.037	0.110	3,116	5.902	0.001
low dose treatment	-0.074	0.084	-0.889	0.376				
Fat	0.117	0.034	3.445	0.001				
FOOD INTAKE								
(Intercept)	0.142	0.029	4.901	0.000				
high dose treatment	-0.005	0.050	-0.108	0.914	0.115	3,116	6.173	0.001
low dose treatment	0.071	0.051	1.407	0.162				
Fat	-0.084	0.021	-4.068	0.000				

Table 6 Outcome of multiple regression models in garden warblers (experiment 2, GHR-test). The dependent variables were Zugunruhe and food intake. Bold typeface represents significant effects

In both the DGHR- and GHR-test, higher fat amount was associated with higher Zugunruhe and lower food intake (*Table 5, 6*).

Discussion

Our results showed that circulating ghrelin reflects the amount of subcutaneous fat reserves in wild migrating garden warblers caught at the stopover site. As far as we know, this is the first study demonstrating that circulating ghrelin levels are a good predictor of the extent of fat reserves. These findings are in line with previous studies in birds showing that ghrelin induces a reduction in lipogenic activity (Buyse et al. 2009; Geelissen et al. 2006), and confirm our first hypothesis that fat deposition might upregulates ghrelin in peripheral tissues. Consequently, ghrelin may trigger the activation of lipid catabolism in the liver, which is required to resume migration. It is worth remembering that fat is the primary fuel during migration and several studies have demonstrated that it is the strongest internal predictor of stopover duration (Fusani et al. 2009; Goymann et al. 2010; Lupi et al. 2016). Furthermore, our results showed a significant effect of the latency, i.e. time between the capture and the plasma collection, on ghrelin levels. In birds, several studies suggest that ghrelin increases adrenocorticotrophic hormone (ACTH) and glucocorticoids through the HPA axis (Furuse et al. 2001; Saito et al. 2002a; Saito et al. 2002b). ACTH and glucocorticoids are known to be secreted during stressful conditions. Moreover, in rats acute stress increases plasma ghrelin levels (Kristensson et al. 2006). All together, these observations indicate that in our birds rapid changing in ghrelin levels according to the latency might be a consequence of the acute stress of the capture.

Our results of the multiple regression models showed that neither peripheral injections of ghrelin or des-acyl ghrelin influence the feeding behaviour. These findings do not support our expectations. In fact, previous studies have demonstrated that endogenous ghrelin, or at least central ghrelin, acts as an anorexigenic neuropeptide in birds (Kaiya et al. 2013; Xu et al. 2011). It remains unclear whether ghrelin directly affects the brain or whether the vagal afferent pathway is involved in ghrelin signalling transmission, as has been demonstrated in rats (Date et al. 2002). However, the effects of ghrelin administered peripherally on food intake seem to vary between bird species and in a dose-dependent manner (Buyse et al. 2009; Geelissen et al. 2006; Kaiya et al. 2007; Ocloń & Pietras 2011; Shousha et al. 2005). Our results are in line with the above findings: although we did not find any significant results, it seems that high doses, but not low doses, of des-acyl ghrelin slightly inhibit food intake. In mammals, des-acyl ghrelin has been reported to be able to either inhibit

(Asakawa et al. 2005; Chen et al. 2005) or stimulate (Koji Toshinai et al. 2006) food intake. With the same high dose of peripherally administered ghrelin, no effect or trend on food intake was seen in our study species. Further studies are required to determine the mode of peripheral des-acyl ghrelin action. So far, it is not possible to measure endogenous des-acyl ghrelin in the blood, as no specific method is at present available.

Our study of the physiology of ghrelin and des-acyl ghrelin in migrating birds is, to our knowledge, the first one to investigate the involvement of this hormone in the regulation of avian migratory behaviour. Particularly during spring migration, several physiological factors are thought to interact in synergy to time the migratory flights and control the behaviour of birds. We asked whether ghrelin and des-acyl ghrelin affect Zugunruhe, as this behaviour shown by nocturnal migrants in captivity is a strong indicator of their migratory disposition (Berthold 1973; Biebach 1985; Eikenaar et al. 2014; Fusani & Gwinner 2004). Several studies demonstrated that in birds exogenous ghrelin administration acts within the brain: central and peripheral ghrelin injections inhibit food intake (Chen et al. 2008; Furuse et al. 2001; Geelissen et al. 2006; Khan et al. 2006; Saito et al. 2005; Shousha et al. 2005) and increase plasma levels of corticosterone, through the involvement corticotropin-releasing factor (CRF) system (Kaiya et al. 2002; Saito et al. 2002). Indeed, the ghrelin cognate receptor GHS-R is widely expressed in many central tissues (Geelissen et al. 2003; Saito et al. 2005; Tanaka et al. 2003). So far, the biological functions of des-acyl ghrelin and its cognate receptors are still unknown. Our results showed that low dose peripheral injections of des-acyl ghrelin increase Zugunruhe in garden warblers. We thus demonstrated that exogenous des-acyl ghrelin induces a dose-dependent increase in migratory disposition. Our results therefore suggest that the stopover decision to resume migration might be triggered by increasing des-acyl ghrelin in the blood. Differently, peripheral injections of ghrelin seem not affecting Zugunruhe. Indeed, though we found that high ghrelin treatment affects Zugunruhe, the effect might hinge on a single outlier value that shows the highest value of Zugunruhe in the control group. Interestingly, these results do not support our expectations relative to the so called ‘active’ ghrelin, but reveal for the first time a physiological function of the des-acyl form of ghrelin in the stopover behaviour of migrating birds.

Overall, our pioneering study provides an initial picture of the role of the hormone ghrelin in the physiology of stopover during migration. Our results suggest that increasing ghrelin in the blood might induce a peripheral stimulation of lipolytic activity, whereas increasing des-acylated ghrelin might centrally increase migratory disposition. The relationship

between the two forms is still not clear (Soares & Moreira, 2008). However, a study demonstrated a strong correlation between des-acyl ghrelin and ghrelin in humans by measuring plasma levels of each of the two forms separately (Akamizu et al. 2005). These observations suggest the existence of a correlation between des-acyl ghrelin and ghrelin in birds. In mammals, it is likely that in the plasma des-acylated ghrelin is produced from ghrelin by deacylation (De Vriese et al. 2007; Shanado et al. 2004) and ghrelin half-life is shorter than des-acyl ghrelin (Soares & Moreira 2008). In the future, it will be important to study the relationship between peripheral ghrelin and lipid catabolism through the measurement of respiratory quotient in migratory birds, and the physiological functions of endogenous des-acyl ghrelin in birds, which requires the validation of a procedure for hormonal measurements.

General Discussion

Migration is still one of the less understood and most fascinating biological phenomena. Its complexity is due to the fact that several exogenous and endogenous factors interact in synergy and the migratory behaviour is the outcome of these interactions. The decision making process about stopover duration is determinant in terms of migratory success, because most of the migration period is spent at stopover sites (Hedenström and Ålerstam 1997), and thus the number and duration of stopovers determines the overall pace of migration. Our research group has been studying the involvement of physiological mechanisms during stopover of small European passerines for several years. My studies illustrate how body condition, feeding behaviour, and the hormone ghrelin participate in the regulation of stopover decision, and complement previous and current studies on the energetics of migratory behaviour. Indeed, physiological mechanisms underlying bird migration are still scarcely known.

During spring migration, our study stopover site, the island of Ponza, is extraordinarily interesting in terms of position along the migratory route. Indeed, short- and long-distance migrants, though characterized by different migratory patterns, have to deal with the same ecological barrier, the Mediterranean Sea, and massive energy expenditure to reach the island. Migratory birds fuel their migratory flights primarily through fatty acid metabolism (McWilliams et al. 2004; Pierce et al. 2005) and previous studies of our group have demonstrated that fat is the strongest internal predictor of stopover duration in long-distance migrants (Fusani et al. 2009; Goymann et al. 2010). Similarly, my studies revealed that in short-distance migrants the departure decision depends on body condition, with individuals in good condition showing higher migratory disposition compared to those in worse condition (Lupi et al. 2016). These findings suggest that birds that faced a large ecological barrier finely adjusted their departure according to body condition. Particularly in spring, birds arriving at a stopover site in poor condition after the crossing of an ecological barrier should be under strong time pressure (Kokko 1999) to reach an optimal fuel load and minimize the duration of the stopover. Depending on which currency the birds are optimizing, e.g. time or energy, different optimal fuel loads at departure are expected (Ålerstam and Lindström 1990). In spring, time minimization is consistently found, especially in long-distance migrants (Lindström and Ålerstam 1992, Dierschke et al. 2005, Maggini and Bairlein 2010). My findings are in line with these observations: in garden warblers, higher fuel deposition rate increases their migratory disposition ('Study 2:

Food intake and stopover decision'). However, in robins fuel deposition rate was not associated with the departure decision, as expected in energy-minimization species. Thus, my results suggest a possible species-specific pattern of departure decisions in response to fuel deposition rates. The position of the stopover site along the migratory route might explain different responses. In fact, it has been shown that migrants left the stopover site quickly and independently from refuelling rate if the stopover site is close to their breeding area (Dierschke and Delingat 2001). Robins caught on Ponza are likely to breed in close locations on mainland Italy (Spina and Volponi 2008) and thus perfectly fit this scenario. Furthermore, my findings show that there is a large component of individual and species-specific variability in food consumption (Lupi et al. submitted). External factors such as food availability, physiological constraints such as digestive tract reduction, and personality traits such as neophobia and differential responsiveness to stressors (e.g. predators, parasite burden) might affect the actual fuel deposition rate. Finally, confounding effects of capture stress in studies involving short-term housing of birds have to be taken into account.

This series of studies add to previous work investigating the energetics of migratory behaviour; however, the physiological mechanism affecting decision making at stopover sites is still unclear. Several hormones are thought to interact in synergy to link the digestive system, the adipose tissue, and the brain through different signalling pathways. My studies show that the hormone ghrelin plays a determinant role in both the metabolic and behavioural response of migrants to the extent of subcutaneous fat reserves, which reflects their general body condition. In fact, fat birds show higher concentrations of ghrelin compared to lean birds, indicating that circulating levels of ghrelin are a good predictor of the extent of fat reserves and suggesting that increasing ghrelin might affect the metabolism through the peripheral stimulation of lipolytic activity. These findings are in line with previous studies in domesticated poultry showing that ghrelin induces a reduction in lipogenic activity in the liver (Buyse et al. 2009; Geelissen et al. 2006). Furthermore, peripheral administration of des-acyl ghrelin, a form of the hormone lacking acyl-modification, increases migratory disposition, suggesting that des-acylated ghrelin might affect migratory behaviour through central stimulation.

Overall, my studies extend our knowledge of the physiological factors involved in decision making during bird migration. Understanding decision-making in migratory animals is of primary importance to predict animal movements and plan wildlife conservation. Our pioneering study on the role of ghrelin in controlling behaviour and metabolism in

migratory birds open new perspectives on the unique physiological adaptations to avian migration.

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