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Avian energy use and its link to oxidative stress

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ABSTRACT

Most organisms require energy for maintenance and functioning of the soma, and the main metabolic pathway for generation of required biochemical energy is *via* aerobic metabolism. Occasionally, animals do encounter situations that necessitate increased levels of energy metabolism but availability of energy is often limited, leading to trade-offs. Some of the common trade-offs, are known as the resource allocation between reproduction-related traits and survival, current and future reproductive success, soma-maintenance and performance traits, or even between maintenance traits. Any given energy allocation may also influence free radical production, since an inevitable consequence of aerobic metabolism is the production of reactive oxygen species (ROS). Antioxidants neutralize ROS but any imbalance in favor of ROS may damage various biomolecules inducing oxidative stress. It is hypothesized that oxidative stress is responsible for the progressive physiological decline of the organisms leading to the aging process, and eventually death. Thus, oxidative stress may act as a cornerstone factor of life-history strategies and has been widely studied in physiological ecology research. In my research, I focused on how oxidative stress is linked to the aerobic demands in birds, and its alteration. Our knowledge pertaining to the effects of energy allocation in different traits on oxidative stress, such as the link between components of energy budget (i.e. thermoregulation, reproductive effort) and somatic-maintenance traits (i.e. antioxidant defense mechanisms) is rather limited and still requires further investigation. Within the framework of my doctoral thesis, I conducted different experimental manipulations that affect components of avian metabolism, and tried to understand the link between energy metabolism and oxidative stress under energetically challenging conditions in two avian species; great tits (*Parus major*) and zebra finches (*Taeniopygia guttata*). I hypothesized that oxidative stress acts both as a cost and a constraint along the lifespan of the organisms, representing a physiological mechanism mediating energy trade-offs between reproductive-

related traits and soma-maintenance. In the first study, I manipulated brood size in a free-living species and compared energy expenditure at the level of daily activities and self-maintenance, and costs at the level of oxidative stress. Females raising enlarged brood increased daily energy expenditure compared to the females raising a natural brood size, but basal metabolic rate was similar for both groups. Even though females tried to compensate the demands of the enlarged broods through increasing energy expenditure for reproductive activities, their nestlings did not develop as fast as nestlings in the control group, perhaps due to increased sibling competition within the brood and energetic limitations of their mothers. Females with higher energy expenditure had lower antioxidant capacity (negative relationship). This came at the cost of higher oxidative stress (positive relationship), indicating that oxidative stress may act as a cost during reproduction. In the second study, I tested heat dissipation limit hypothesis, assuming that the ability to dissipate body heat produced during intensive workload may constrain animals from sustaining performance at high levels. I manipulated the capacity to dissipate heat in breeding zebra fiches through exposure to a cold (14 °C) or warm (25 °C) ambient temperature and later *via* a feather-clip manipulation around the brood patch in mothers, while I estimated oxidative stress before reproduction, during the peak and towards the end of food-provisioning. Females with the experimentally enhanced possibility to dissipate heat lost less body mass and raised heavier and larger offspring than the non-manipulated females. This suggests that the ability to dissipate more heat allowed females to invest more energy towards reproductive output without constraining self-maintenance, in line with the heat dissipation limit hypothesis. Even though feather-clip manipulation and ambient temperature had no effect on oxidative stress during reproduction, the antioxidant capacity decreased significantly towards the end of reproduction. This outcome corroborates the hypothesis that reproduction is costly in terms of oxidative stress. The present thesis provides evidence that the capacity to heat dissipate indeed acts as a constraint during intensive workload, especially

under warm conditions, and oxidative stress may act as both cost and constraint during reproductive activities. Reproductive effort may come with lower antioxidant capacity and this may increase the risk of encountering oxidative stress. Thermal limitations can be expected to impose even bigger and more strenuous challenges for organisms in general, and during reproduction in particular, while oxidative stress avoidance during reproduction might be the key to understand the allocation of resources to either current or future reproduction.

STRESZCZENIE

Większość organizmów potrzebuje energii do utrzymania i funkcjonowania somy, a głównym szlakiem metabolicznym do wytworzenia potrzebnej energii biochemicznej jest metabolizm tlenowy. Zwierzęta napotykają sytuacje, które wymagają zwiększonego zużycia energii, ale dostępność energii jest często ograniczona, co prowadzi do kompromisów ewolucyjnych. Powszechnie znanymi kompromisami są na przykład alokacja zasobów między cechami związanymi z reprodukcją a przeżywalnością, obecnym i przyszłym sukcesem reprodukcyjnym, utrzymaniem somy i cechami wydolnościowymi, a nawet między różnymi cechami odpowiedzialnymi za utrzymanie somy. Każda alokacja energii może również wpływać na produkcję wolnych rodników tlenowych, ponieważ nieuniknioną konsekwencją metabolizmu tlenowego jest produkcja reaktywnych form tlenu (RFT lub ROS od ang. reactive oxygen species). Przeciwutleniacze neutralizują RFT, ale zaburzenie równowagi w kierunku RFT może prowadzić do uszkodzenia biocząsteczek powodując stres oksydacyjny. Przypuszcza się, że stres oksydacyjny jest odpowiedzialny za postępujące obniżenie fizjologicznej wydolności organizmów, prowadząc do procesu starzenia, a ostatecznie do śmierci. Stres oksydacyjny może działać więc jako fundamentalny czynnik wpływający na historie oraz strategie życiowe i był obszernie badany w ramach ekologii fizjologicznej. W swoich badaniach skupiłam się na pytaniu w jaki sposób stres oksydacyjny jest związany z zapotrzebowaniem energii u ptaków oraz czy ta zależność może zostać zmieniona. Nasza wiedza na temat wpływu alokacji energii w różnych cechach na stres oksydacyjny, takich jak związek między składnikami budżetu energetycznego (tj. termoregulacja, wysiłek reprodukcyjny) a cechami somatyczno-konserwacyjnymi (tj. mechanizmami obrony antyoksydacyjnej), jest dość ograniczona i nadal wymaga dalszych badań. W ramach mojej pracy doktorskiej przeprowadziłam różne eksperymentalne manipulacje, które wpłynęły na komponenty metabolizmu ptaków i próbowałam zrozumieć związek między metabolizmem

energetycznym a stresem oksydacyjnym w trudnych energetycznie warunkach u dwóch gatunków ptaków; bogatka zwyczajna (*Parus major*) i zeberka australijska (*Taeniopygia guttata*). Postawiłam hipotezę, że stres oksydacyjny działa zarówno jako koszt, jak i ograniczenie w ciągu życia organizmów, reprezentując mechanizm fizjologiczny pośredniczący w kompromisach energetycznych między cechami związanymi z reprodukcją a utrzymaniem somy. W pierwszym badaniu manipulowałam wielkością lęgu u gatunku wolnożyjącego i porównałam wydatek energetyczny na poziomie codziennych aktywności i utrzymania własnego organizmu, oraz koszty na poziomie stresu oksydacyjnego. Samice wychowujące powiększone lęgi podniosły dobowy wydatek energetyczny w porównaniu z samicami wychowujących lęgi naturalnej wielkości, natomiast podstawowe tempo metabolizmu było podobne w obu grupach. Mimo, że samice próbowały skompensować zwiększone wydatki energetyczne powiększonych lęgów poprzez zwiększenie wydatków energetycznych na czynności reprodukcyjne, ich pisklęta nie rozwijały się tak szybko jak pisklęta z grupy kontrolnej. Może to być związane ze zwiększoną konkurencją między rodzeństwem w gnieździe i ograniczeniami energetycznymi ich matek. Samice o wyższym wydatku energetycznym miały niższą wydolność antyoksydacyjną (zależność negatywna), co było powiązane z kosztem wyższego stresu oksydacyjnego (zależność pozytywna), i wskazuje, że stres oksydacyjny może działać jako koszt podczas reprodukcji. W drugim badaniu przetestowałem hipotezę limitu rozpraszania ciepła zakładającą, że zdolność rozpraszania ciepła ciała wytwarzanego podczas intensywnego wysiłku może ograniczać zwierzęta do utrzymania sprawności na wysokim poziomie. Manipulowałam zdolnością do rozpraszania ciepła u hodowlach zeberek poprzez wystawienie je na działanie zima (14°C) lub ciepła (25°C), a później poprzez manipulację strzyżeniem piór wokół łaty lęgowej u matek. Oszacowałam stres oksydacyjny przed rozrodem, w szczytowym okresie zaopatrzenia w pokarm pisklątoraz pod jego koniec. Samice o eksperymentalnie zwiększonej możliwości rozpraszania ciepła

traciły mniej masy ciała i wychowywały cięższe i większe potomstwo niż samice niemanipulowane. Sugeruje to, że zdolność do rozpraszania większej ilości ciepła pozwalała samicom inwestować więcej energii w reprodukcję bez ograniczania utrzymania własnej somy zgodnie z hipotezą limitu rozpraszania ciepła. Mimo, że manipulacja strzyżeniem piór i temperaturą otoczenia nie miały wpływu na stres oksydacyjny podczas reprodukcji, zdolność antyoksydacyjna znacznie spadła pod koniec reprodukcji. Wynik ten potwierdza hipotezę, że reprodukcja jest kosztowna pod względem stresu oksydacyjnego. Niniejsza rozprawa dostarcza dowodów, że zdolność do rozpraszania ciepła rzeczywiście działa jako ograniczenie podczas intensywnego obciążenia wysiłkiem, zwłaszcza w ciepłych warunkach, a stres oksydacyjny może działać zarówno jako koszt, jak i ograniczenie podczas czynności reprodukcyjnych. Wysiłkowi rozrodczemu może towarzyszyć niższa zdolność antyoksydacyjna, co może zwiększać ryzyko wystąpienia stresu oksydacyjnego. Można oczekiwać, że ograniczenia termiczne będą stanowić jeszcze większe i bardziej uciążliwe wyzwania dla organizmów w ogóle, a w szczególności w okresie reprodukcji, podczas gdy unikanie stresu oksydacyjnego w czasie reprodukcji może być kluczem do zrozumienia alokacji zasobów między obecnym lub przyszłym rozmnażaniem się.

THESIS STRUCTURE

The dissertation is divided into three chapters. The first chapter (CHAPTER I) provides a general background and introduction to life-history trade-offs, energetic limitations and strategies during reproduction, oxidative stress and finally the integration between energetic parameters and oxidative stress during avian reproduction. The following chapter (CHAPTER II) includes the description of the two experiments, Experiment I and Experiment II, that were conducted for answering the research questions of the present thesis. The description of each study features a brief summary, followed by a detailed description of the methodological procedures, and is concluded with presentation of the results of the study. The final chapter (CHAPTER III) includes the discussion of the results of both experiments and the conclusion, presenting in addition some of the perspectives that arise from this PhD work.

ABBREVIATIONS

BMR	Basal metabolic rate	Represents the amount of energy that an organism requires for maintaining basic process to self-maintain and it is typically measured when an animal is resting, non-reproducing and post-absorptive under thermoneutral conditions. Units: Watt (Joule/Second) or ml O_2/min
RMR	Resting metabolic rate	Represents the minimum amount of energy that an organism requires for self-maintenance under resting although outside thermoneutral conditions. Units: Watt (Joule/Second) or ml O_2/min
DEE	Daily energy expenditure	Represents the total amount of energy over a 24- hour period expended in performance, thermoregulation and regular processes of soma-maintenance. Units: Watt (Joule/Second)
SusMS	Sustained metabolic scope	Represents the maximum power output committed to activities other than maintenance needs, measured during sustained period of workload, calculated as the ratio between daily energy expenditure and basal metabolic rate (SusMS = DEE/BMR).
DLW	Doubly labelled water	This is a technique that uses stable isotopes of hydrogen and oxygen in animals to trace the flow of water and carbon dioxide through the body over time, to estimate daily energy expenditure.
TNZ	Thermoneutral zone	A thermal comfort zone with a range of ambient temperatures in which endothermic organisms spend minimum energy for thermal homeostasis.
HDL	Heat dissipation limit	Heat dissipation limit hypothesis poses that the capacity to dissipate the excess of body heat during hard work may limit sustained energy use.
ROS	Reactive oxygen species	Molecules derived from O_2 that are very unstable and react very fast with anything in their surrounding environment, causing oxidative damage to biochemical structures and molecules.
ROMs	Reactive oxygen metabolites	ROMs include mostly hydroperoxides that are early peroxidation products when ROS interact with many different biological macromolecules.

d-ROMs	d-ROMs test	A common test usually performed to measure reactive oxygen metabolites (ROMs) in plasma that include mostly hydroperoxides.
OXY	OXY-adsorbent test	This test was performed to estimate total non-enzymatic antioxidant capacity in plasma.

CHAPTER I General Introduction

"*Since an organism is inseparable from its environment, any person who attempts to understand an organism's distribution must keep in mind that the item being studied is neither a stuffed skin, a pickled specimen, nor a dot on a map. It is not even the live organism held in the hand, caged in the laboratory, or seen in the field. It is a complex interaction between a self-sustaining physicochemical system and the environment*."

G. Bartholomew, 1958

1.1. Energetic boundaries and trade-offs during reproduction

Life-history theory attempts to explain how natural selection and other evolutionary forces shape organisms in order to maximize their survival and reproduction in the presence of environmental challenges (Stearns, 1992, 2000; Roff, 1993). The concept of life-history theory is based on the notion that organisms have limited amount of resources to allocate and thus often encounter trade-offs. One of the most studied and common trade-offs occurs during reproduction, when parents are challenged to invest energy either towards current reproductive outcome or somatic-maintenance and thus future reproduction or survival (Zera and Harshman, 2007; Santos and Nakagawa, 2012; Garland et al., 2022). Life-history trade-offs are often assumed to have a physiological basis (Zera and Harshman, 2007; Monaghan et al., 2009). Therefore, there is vast body of research and a continuous effort in the field of energetics for understanding the link between energy management and life-history traits - integrating the interfaces of ecology, evolutionary biology, biochemistry and physiology (Carey, 1996).

Energy is fundamental for all living organisms and life-history decisions are primarily influenced by the energy supply and use (Weiner, 1992). However, energy is limited and animals are not always able to maximize their energy expenditure for a sustained period, and thus frequently encounter energetic boundaries (Drent and Daan, 1980; Peterson et al., 1990; Hammond and Diamond, 1997; Piersma, 2011). For instance, birds along the annual cycle very often face challenging conditions that require particularly high levels of energy expenditure (such as reproduction, migration etc.). Because of the underlying constrains, during energetically challenging conditions organisms the energy use may be traded-off between two or more competing demands (Stearns, 1992; Roff, 1993). Such energy allocation between individual traits is also often assumed to have negative consequences on other traits (i.e. oxidative stress), and in this thesis I will investigate energy allocation and potentially associated trade-offs and costs during avian reproduction.

During reproduction, birds use energy to establish a territory, find a mate, build a nest, lay and incubate eggs, and finally to raise their offspring. Usually, the energetic costs of the breeding adults are investigated by common measures of energy expenditure that include daily energy expenditure (DEE), basal metabolic rate (BMR) or by experimentally manipulating parental effort, i.e. enlarging the brood size (Bouwhuis et al., 2011; Santos and Nakagawa, 2012). While DEE comprises the total amount of energy expended on behaviour, thermoregulation and maintenance processes, BMR represents the minimum energetic costs of tissue and organ maintenance (Ricklefs 1996 in Carey 1996; Bouwhuis, Sheldon, and Verhulst 2011). Offspring rearing period requires increased physical activity, and individuals capable of sustaining higher energy expenditure may lay larger clutches or raise offspring in better condition (Moreno et al., 1997). If such a positive correlation exists between parental energy expenditure and reproductive success, breeding adults may adjust different energy management strategies to meet the energetic requirements of parental care (Daan et al., 1990a; Ellison, 2003; Portugal et al., 2016). Since there is a considerable variation in BMR and DEE within and between individuals, and both components represent energetic costs (Speakman et al., 2003; Welcker et al., 2015) it has been suggested that BMR contributes to sustained levels of DEE (Vézina et al., 2006). Generally, BMR represents around 30 to 40% of the total daily energy budget (Speakman, 2000) and adults may work at levels four times that of BMR during offspring-provisioning period (Drent and Daan, 1980). It is assumed that during such high energetic demands organisms may allocate energy between the different reproductive activities (i.e. feeding their nestlings) and the requirements for self-maintenance, but DEE may be constrained from BMR adjustments for maximizing energy expenditure (Daan et al., 1990b; Ricklefs et al., 2006; Vézina et al., 2006; Burton et al., 2011). The link between BMR and DEE and whether DEE is constrained by BMR for maximizing energy expenditure (Daan et al., 1990b; Nilsson, 2002; Ricklefs et al., 2006; Vézina et al., 2006; Burton et al., 2011; Visser et al., 2019) or BMR

is adjusted to requirements represented by DEE is still unclear (Ricklefs in Carey 1996). The direction of such association can be either positive or negative and two hypotheses have been promoted as energy management strategies that an animal may operate during intensive daily workloads (Nilsson, 2002).

First hypothesis known as the "increased-intake hypothesis" implies a positive relationship between DEE and BMR, meaning that high DEE requires high metabolic machinery (Hammond and Diamond, 1997; Nilsson, 2002). For example, marsh tits (*Puerile palustris*) increased both DEE and BMR when faced high workload, induced by a brood enlargement, to meet the energetic needs during the chick rearing period (Nilsson, 2002). Second hypothesis, the "compensation hypothesis", suggests that maintaining a high BMR can be energetically costly and thus, an animal with high BMR would have less surplus energy to use for other energetic demanding activities such as food provisioning (Deerenberg et al., 1998; Nilsson, 2002). Since sustained energy expenditure is limited (Peterson et al., 1990; Hammond and Diamond, 1997), a rather negative relationship should be observed between DEE and BMR with animals keeping BMR at low levels increasing the available amount for daily activities ("compensation hypothesis": see Johnston et al. 2007; Burton et al. 2011; Welcker et al. 2015; Visser et al. 2019). Even though the relationship between DEE and BMR has been previously explored both in mammals and birds (Meerlo et al., 1997; Fyhn et al., 2001; Speakman et al., 2003; Vézina et al., 2006; Careau et al., 2013; Welcker et al., 2015; Visser et al., 2019), the direction of such causality is still unclear. At the end, one may suggest that both vary in parallel and each energetic investment may entail costs or benefits to the organism and largely depend on energetic limitations that the organism may encounter.

Energy limitations during sustained energy expenditure can be imposed by either external (e.g. food availability; Speakman et al., 2003), or internal physiological and morphological factors (Drent and Daan, 1980; Hammond and Diamond, 1997; West et al., 1999). As an external

factor, the availability of food resources has been suggested to constrain performance since energy expenditure needs to be refuelled. Although studies under *ad libitum* food availability cannot always explain energy limitation (see meta-analysis Boutin, 1990) implying that rather intrinsic physiological factors constrain sustained energy expenditure, several studies have also shown that habitats with high food abundance may increase the rate of energy expenditure (Speakman et al., 2003; Welcker et al., 2009; Jodice et al., 2006). Another hypothesis that was later formulated and tested suggests that sustained energy expenditure is limited by the capacity of the alimentary tract to process and digest the incoming energy, known as the "central limitation hypothesis" (Drent and Daan, 1980; Weiner, 1992; Koteja, 1996; Thurber et al., 2019). For instance, hummingbirds spent most of the time perching despite their high energetic requirements, perhaps waiting for the digestion process and only after emptying their crop they could maximize energy, indicating that the capacity to digest may act as an energetic constrain (Diamond et al., 1986). "Metabolic theory of ecology" suggests that the rate at which the circulatory system can deliver nutrients to organs and tissues limits energy (West et al., 1999). On the other hand, "peripheral limitation" hypothesis posits that the efficiency of the organs themselves may constrain maximum and sustained work rate, such as the skeletal muscles to produce physical work, or the mammary glands to produce milk (Hammond and Diamond, 1997). Lastly, heat dissipation limit (HDL) hypothesis proposes that the individual's capacity to dissipate the excess of body heat generated during hard work may actually limit the use of energy (Speakman and Król, 2010).

A number of empirical studies in mammals indicate that the problem to dissipate heat may indeed constrain individuals to perform at high and sustained levels of energy expenditure. First observations of cold exposure, thus higher heat loss rate from the body, revealed that when mice (outbred MF1, *Mus musculus*) were exposed to ambient temperature of 8 °C during lactation (most energetic demanding phase), produced more milk and raised heavier offspring

compared to mice that were exposed to a higher ambient temperature of 21 °C (Johnson and Speakman, 2001; Król et al., 2007). In birds, even though the heat production is not directly associated with intrinsic processes such as in lactating mammals, nestling-rearing requires an intensive physical workload for food provisioning. Zebra finches (*Taenopygia guttata*) raised offspring bigger in structural size and heavier in mass (when 28 days old) when exposed to 14 °C than the zebra finches exposed to 30 °C (Andrew et al., 2017). Further studies support HDL by manipulating and enhancing heat dissipation via fur removal in mammals (Król et al., 2007), or feather-clipping in birds (Nord and Nilsson, 2019; Tapper et al., 2020b). Increased ability to dissipate heat via a feather-clip manipulation in female blue tits (*Cyanistes caeruleus*) seemed to have relaxed limitations compared to un-manipulated control birds (Nord and Nilsson, 2019); feather-clipped females could minimize body mass loss compared to controls and also raised heavier nestlings (Nord and Nilsson, 2019). Feather-clipped tree swallows (*Tachycineta bicolor*) raised nestlings with higher body mass and maintained higher workload and lower day-time body temperature than the control ones (Tapper et al., 2020b, 2020a). Those effects of ambient temperature and treatment with increased heat loss rate in both mammals and birds support the idea of HDL in endotherms (although see for humans Thurber et al., 2019 and Rogowitz, 1998; Yang et al., 2013), resulting in high reproductive performance and output. So far, evidence supporting HDL hypothesis in birds exists only under field conditions while experimentally testing HDL under laboratory studies in birds are yet to be implemented.

1.2. Oxidative stress

Resource allocation between different components during reproduction may also impact other traits and have negative consequences (Zera and Harshman, 2007; Monaghan et al., 2009). For example, oxidative stress is considered to act as a physiological cost or even constraint during

life-history trade-offs (Monaghan et al., 2009). Increasing investment towards reproductive output may also carry costs causing somatic damage (Harshman and Zera, 2007) eventually affecting survival and future reproduction. Oxidative stress may result from an imbalance between reactive oxygen species and the antioxidants (Figure 1; Jones 2006; Sies 1997). Regular processes of aerobic metabolism generate a variety of reactive oxygen species (ROS) oxygen-derived (O_2), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydrogen radicals ($OH^{\bullet}$) (Frisard and Ravussin, 2006). Due to their unpaired electron, those molecules are very unstable and react very fast with practically any other chemical species in their surroundings, causing oxidative damage to biochemical structures and molecules, such as lipids, proteins and nucleic acids (Beckman and Ames, 1998). However, organisms under normal conditions employ different ways to maintain oxidative balance (Figure 1). The first line of protection tries to minimize ROS production within cells, either through membrane composition or uncoupling of oxygen consumption and ATP production (Monaghan et al., 2009; Alan and McWilliams, 2013). Antioxidant defence system comes as a second line of protection with antioxidant enzymes or metal-binding proteins to counteract ROS, and plays a significant role in protection against oxidative stress (Ji, 1999; Jones, 2006; Monaghan et al., 2009). Antioxidants may occur in enzymatic and non-enzymatic form in the intracellular and extracellular environment (Nimse and Pal, 2015). A number of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) together with other metal binding ions (i.e. manganese, iron, zinc and copper) are responsible for converting free oxygen radicals into hydrogen peroxide and ultimately to water. Non-enzymatic antioxidants such as glutathione, vitamin E, vitamin C, carotenoids, uric acid that are naturally endogenously synthesized or obtained through diet are binding with ROS terminating further chain reaction. A final line of defence takes place if some oxidative damage will still occur despite the above mentioned antioxidant defences by removing or repairing damaged

biomolecules (Monaghan et al., 2009). When organisms are exposed to situations and elements that influence metabolic processes, like induced physical activity or changes in the ambient temperature, ROS production increases, and maintaining an oxidative balance can be a challenge.

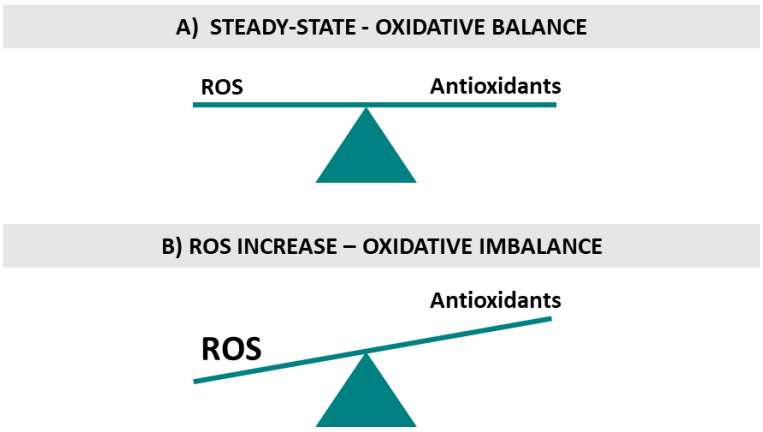

Figure 1. A schematic illustration during A) normal physiological conditions, where oxidative balance is maintained between ROS and antioxidants and B) oxidative imbalance when ROS production exceeds the antioxidant capacity for a certain time (adjusted from Monaghan et al., 2009).

1.3. Oxidative stress during avian reproduction

Oxidative stress during reproduction may represent a) a physiological cost ("oxidative cost hypothesis"), b) a constrain ("oxidative constrain hypothesis"), or c) a shielding ("oxidative shielding hypothesis") (Viblanc et al., 2018; Meniri et al., 2022). Since reproduction is often energetically demanding with increased physical activity for offspring-provisioning it may enhance ROS production (Monaghan et al., 2009). Several studies have attempted to explore the effects of increased energy metabolism and different measurements of oxidative status during reproduction in both mammals and birds (see reviews Stier et al., 2012; Metcalfe and

Monaghan, 2013) but here, I mainly discuss and focus on existing studies in birds (see Table 1).

Initial studies showed that higher reproductive effort via a brood enlargement comes with lower antioxidant capacity (Alonso-Alvarez et al., 2004; Wiersma et al., 2004). In line with this pattern, Seychelles warblers (*Acrocephalus sechellensis*) and female barn swallows (*Hirundo rustica*) exhibited lower antioxidant capacity during food provisioning period compared to early reproduction stage (van de Crommenacker et al., 2012; Costantini et al., 2014c). Rearing offspring is demanding in terms of energy and time, and breeding adults may not be able to invest enough for self-maintenance, i.e. by self-feeding and hence increasing antioxidant capacity through exogenous antioxidants. Such decline in antioxidants may reflect the need to protect from oxidative damage during high workloads (Costantini, 2011). Contrary to these findings, nestling visiting rate, a proxy of energy expenditure, was positively related to antioxidant enzymes (Casagrande and Hau, 2018). Increased energy expenditure may not necessarily lead to a uniform pattern of the antioxidant defence mechanisms indicating that different strategies may be employed to cope with ROS produced due to high energetic requirements of reproduction.

Current knowledge on the relationship between reproduction and oxidative stress in birds is rather complex as evident in Table 1, which lists different studies regarding the link between reproduction and oxidative stress. Oxidative stress is usually documented through measurements of common markers of oxidative stress either in plasma or in erythrocytes such as oxidative damage at the early phase of oxidative cascade (d-ROMs) or total non-enzymatic antioxidant capacity (OXY) in plasma, resistance of red blood cell membranes to an oxidative burst (KRL test), and less frequently enzymatic antioxidants or uric acid in plasma. These biomarkers are usually estimated during early reproduction (i.e. incubation phase) or during the peak of food provisioning period (19 out of 34 studies presented in Table 1) as the most

energetically demanding period, while repeated measurements within-individual before and along the reproduction is less frequent (4 out 34 cases; see Table 1). Indeed, a repeated with-individual approach could actually disentangle the role of oxidative stress (constrain, cost or shielding) during the biology of reproduction (Stier et al., 2012), although it is hardly measured under field conditions due to technical obstacles. As observed in Table 1, most studies are based on the manipulation of reproductive status, breeding versus non-breeding animals, or reproductive effort by comparing brood size manipulation groups, enlarged versus control broods. For instance, higher oxidative damage was not detected in breeding individuals compared to non-breeding individuals (Costantini et al., 2014b) contrary to the expected positive link between metabolism and free radical production. However, when oxidative stress is measured and tested in relationship to the given breeding status or the brood size (number of offspring), the energy expenditure is hardly measured at the level of daily performance or/and at the level of self-maintenance. For instance only one study measured daily energy expenditure under a brood size manipulation and the antioxidant capacity (Wiersma et al., 2004). While other studies estimated (3 out 34) feeding rate which is often assumed as a proxy of energy expenditure during reproduction (Costantini et al., 2014a; Cram et al., 2015; Casagrande and Hau, 2018), those measurements do not reflect energy expenditure at the level of self-maintenance (Vaanholt et al., 2008; Bury et al., 2018). Given the fact that any changes in ROS production or responses of the antioxidant mechanisms are highly triggered by the aerobic metabolism *per se*, many of the contradictions of these studies could be resolved by providing a comprehensive information of the oxidative status in conjunction with energy metabolism for self-maintenance and daily costly activities.

In order to gain a better understanding of the interplay among reproductive activities, oxidative stress and reproductive output, I conducted two studies; one under field conditions measuring several energetic parameters and markers of oxidative stress during the peak of food

provisioning period and one under laboratory conditions measuring oxidative stress several times on different stage of reproduction.

Table 1. A summary of existing studies in birds regarding the link between reproduction and oxidative stress (OS) biomarkers. The table indicates a) the species name of the study, b) the design of the experiment whether it took place under field or laboratory conditions, c) manipulation of the study that influence reproductive effort, d) the marker of oxidative stress categorized separately, e) the blood sampling period; before reproduction, early phase during incubation or during peak provisioning period, f) the tissue sampled for the oxidative stress biomarker, g) the sex of the individual (F: Female, M: Male), h) the reproductive trait that was measured and tested in relationship with the given OS biomarker and i) the relationship between reproductive trait and the OS biomarker (if the study reports results different for each sex, the relationship is shown for both sexes separated by a slash /).

Species	Experiment	Manipulation of the study	OS biomarker	Sampling period	Sample type	Sex	Energetic Parameter	Reproductive trait	Relationship	Reference
Common starling (*Sturnus vulgaris*)	field	none	d-ROMs	early	plasma	F	−	brood size	+	(Costantini et al., 2010)
Great tit (*Parus major*)	field	none	d-ROMs	early	plasma	F	−	brood size	0	(Costantini et al., 2010)
Seychelles warbler (*Acrocephalus sechellensis*)	field	malaria infection	d-ROMs	before/early/during	plasma	F/M	−	status	+**	(van de Crommenacker et al., 2012)
Adelie penguins (*Pygoscelis adeliae*)	field	handicap*	d-ROMs	during	plasma	F/M	−	effort	0 / 0	(Beaulieu et al., 2011)
Canary (*Serinus canaria*)	laboratory	brood size	d-ROMs	during	plasma	F/M	−	status	−	(Costantini et al., 2014b)
Canary (*Serinus canaria*)	laboratory	OS induced	d-ROMs	early	plasma	F	−	laying date, clutch size	−	(Costantini et al., 2016)
Collared flycatcher (*Ficedula albicollis*)	field	none	d-ROMs	early	plasma	F	−	clutch size	0	(Markó et al., 2011)
European kestrel (*Falco tinnunculus*)	field/captive	none	d-ROMs	early/during	plasma	F/M	−	status	0 / +	(Casagrande et al., 2011)
Great tit (*Parus major*)	field	handicap	d-ROMs	early/during	plasma	M	feeding rate	effort	0	(Casagrande and Hau, 2018)
Barn swallow (*Hirundo rustica)*	field	brood size	d-ROMs	early/during	plasma	F/M	feeding rate	effort	0	(Costantini et al., 2014a)
Zebra finch (*Taeniopygia guttata*)	laboratory	brood size, 10 °C	d-ROMs	before/during	plasma	F/M	−	effort	0	(Sudyka et al., 2016)
Common starling (*Sturnus vulgaris*)	field	none	OXY	early	plasma	F	−	brood size	−	(Costantini et al., 2010)
Great tit (*Parus major*)	field	none	OXY	early	plasma	F	−	brood size	−	(Costantini et al., 2010)
Seychelles warbler (*Acrocephalus sechellensis*)	field	malaria infection	OXY	before/early/during	plasma	F/M	−	status	−	(van de Crommenacker et al., 2012)

Species	Experiment	Manipulation of the study	OS biomarker	Sampling period	Sample type	Sex	Energetic Parameter	Reproductive trait	Relationship	Reference
Adelie penguins (*Pygoscelis adeliae*)	field	handicap*	OXY	during	plasma	F/M	−	effort	+ / +	(Beaulieu et al., 2011)
Collared flycatcher (*Ficedula albicollis*)	field	none	OXY	early	plasma	F	−	clutch size	0	(Markó et al., 2011)
European kestrel (*Falco tinnunculus*)	field/captive	none	OXY	early/during	plasma	F/M	−	status	+ / 0	(Casagrande et al., 2011)
Great tit (*Parus major*)	field	handicap	OXY	early/during	plasma	M	feeding rate	effort	0	(Casagrande and Hau, 2018)
Barn swallow (*Hirundo rustica)*	field	brood size	OXY	early/during	plasma	F/M	feeding rate	effort	− / 0	(Costantini et al., 2014a)
Zebra finch (*Taeniopygia guttata*)	laboratory	brood size, 10 °C	OXY	before/during	plasma	F/M	−	effort	−	(Sudyka et al., 2016)
Florida scrub jay (*Aphelocoma coerulescens*)	field	none	protein carbonyl	before/after	plasma	F/M	−	effort	0 / +	(Heiss and Schoech, 2012)
Canary (*Serinus canaria*)	laboratory	brood size	protein carbonyl	during	plasma	F/M	−	status	−	(Costantini et al., 2014b)
Zebra finch (*Taeniopygia guttata*)	laboratory	carotenoid supplementation	resistance	before/after	erythrocyte	F/M	−	status, number of eggs	− / −	(Bertrand et al., 2006)
Great tit (*Parus major*)	field	brood size	resistance	during	erythrocyte	M	−	effort	−	(Losdat et al., 2011)
Great tit (*Parus major*)	field	brood size, malaria infection	resistance	during	erythrocyte	F/M	−	effort	−	(Christe et al., 2012)
Zebra finch (*Taeniopygia guttata*)	laboratory	brood size	resistance	during	erythrocyte	F/M	−	effort	− / −	(Alonso-Alvarez et al., 2004)
Alpine swift (*Apus melba*)	field	cross-foster	resistance	early	erythrocyte	F/M	−	hatchling success	+ / 0	(Bize et al., 2008)
Alpine swift (*Apus melba*)	field	cross-foster	resistance	early	erythrocyte	F/M	−	clutch size	+ / 0	(Bize et al., 2008)
Adelie penguins (*Pygoscelis adeliae*)	field	handicap*	uric acid	during	plasma	F/M	−	effort	0 / 0	(Beaulieu et al., 2011)
White-browed sparrow weaver (*Plocepasser mahali*)	field	clutch removal	uric acid	early/during	erythrocyte	F/M	feeding rate	effort	0	(Cram et al., 2015)
Zebra finch (*Taeniopygia guttata*)	laboratory	brood size	antioxidant enzymes	during	pectoral muscle	F/M	DEE	effort	− / −	(Wiersma et al., 2004)

Species	Experiment	Manipulation of the study	OS biomarker	Sampling period	Sample type	Sex	Energetic Parameter	Reproductive trait	Relationship	Reference
			(SOD, GPx)							
Great tit (*Parus major*)	field	handicap	GPx	early/during	erythrocyte	M	feeding rate	effort	+	(Casagrande and Hau, 2018)
Canary (*Serinus canaria*)	laboratory	OS induced	GSH	early	erythrocyte	F	−	hatching success, fledging success, brood size	0	(Costantini et al., 2016)
White-browed sparrow weaver (*Plocepasser mahali*)	field	clutch removal	MDA	early/during	plasma	F/M	feeding rate	effort	+	(Cram et al., 2015)
White-browed sparrow weaver (*Plocepasser mahali*)	field	clutch removal	SOD	early/during	erythrocyte	F/M	feeding rate	effort	0	(Cram et al., 2015)
White-browed sparrow weaver (*Plocepasser mahali*)	field	clutch removal	TAC	early/during	erythrocyte	F/M	feeding rate	effort	0	(Cram et al., 2015)

* the study refers to a handicap manipulation due to the attachment of a device to increase locomotion costs but it does not necessary affect reproductive effort
** The effect only is observed for the malaria infected individuals

1.4. Aim of the thesis

Even though intensively studied, the effect of reproduction on oxidative stress remains unresolved. My thesis aims at understanding how oxidative stress is linked to the aerobic demands of avian reproduction under energetic challenging conditions. I manipulated reproductive effort either by enlarging brood size (**Experiment I**) or by enhancing the ability to dissipate heat (**Experiment II**) and measured several energetic parameters during reproduction to estimate the resource allocation between different reproductive and fitness related traits under experimentally increased workload and the relative costs in terms of oxidative stress.

As a first step (**Experiment I**), I conducted a field experiment on great tits (*Parus major*), a hole-nesting passerine which is commonly used as an avian model species in ecology (Gosler, 1993), during nestling-rearing period. For **Experiment I**, I sought to investigate how much basal metabolic rate contributes to daily energy expenditure during sustained workload, by experimentally enhancing workload via a brood enlargement. To identify the costs of reproduction in oxidative stress, I measured several oxidative stress biomarkers in plasma: oxidative damage at the early phase of oxidative cascade (d-ROMs), total non-enzymatic antioxidant capacity (OXY), uric acid (a potent antioxidant in birds), and I estimated oxidative stress index (d-ROMs/OXY x 1000). I analysed the differences in energy expenditure, oxidative damage, antioxidant capacity and reproductive output between females raising enlarged broods and control broods. Final aim was to investigate the relationship between the different energetic components and oxidative stress for the same individuals.

As for **Experiment II,** I aimed at testing heat dissipation limit (HDL) hypothesis in zebra finches (*Taeniopygia guttata*) by experimentally enhancing the ability to release heat either

through exposure to a cold or warm ambient temperature or *via* manipulation of feather insulation and investigated the effects on oxidative stress. I measured several components of energy expenditure (i.e. food intake, body mass) and reproductive traits (i.e. egg mass, clutch size, hatchlings etc.) along the reproduction to quantify reproductive effort and output. I estimated oxidative status of the females a) before ("constrain hypothesis"), b) during and c) towards the end of reproduction ("cost hypothesis") through measuring different biomarkers in plasma: d-ROMs, OXY, uric acid and finally oxidative stress index (d-ROMs/OXY x 1000). I compared reproductive effort (i.e. food intake), self-maintenance (body mass) and oxidative status between the mothers with the possibility to loose heat and un-manipulated control mothers and their reproductive output (i.e. egg production, nestling development etc.).

1.5. Study systems and traits of interest

For **Experiment I**, I studied the great tit (*Parus major*) species, a hole-nesting passerine and very common study system in ecology (Gosler, 1993). Great tits can be found over a broad geographical range from Europe to Asia and some northern parts of Africa (Gosler, 1993). They breed commonly in nest boxes and are tolerant to frequent human presence (and handling) during breeding period. Therefore, I selected great tit species, a species easy to catch, handle and to take various measurements to quantify physiological mechanisms during reproduction under natural conditions.

Generally, the egg-laying phase in the Western Palearctic region starts in the middle of April and the clutch size in great tit species ranges from 7 to 11 eggs (Pettifor et al., 2001). I conducted a brood size manipulation to experimentally increase reproductive effort, as it is commonly performed in great tits (Sanz and Tinbergen, 1999; Tinbergen and Verhulst, 2000; Hõrak, 2003; Wiersma and Tinbergen, 2003; Wegmann et al., 2015), one day after egg-hatching. The peak of food provisioning is considered as sustained workload period when

nestlings reach 9 to 11 days old, since adults have been working hard for an extended time (Drent and Daan, 1980). Using doubly-labelled water (DLW) method, I estimated daily energy expenditure (DEE) to quantify energy expenditure over a 24-hour period as a measure of sustained metabolic rate, during the peak of food provisioning in great tit females. I also estimated energetic costs of self-maintenance during resting and under thermoneutral conditions, basal metabolic rate (BMR). Both DEE and BMR allowed me to estimate sustained metabolic scope (SusMS = DEE/BMR), which typically represents the amount of energy budget committed to activities other than maintenance needs (Pörtner et al., 2017; Buttemer et al., 2019). Only few data exist in the avian literature on such a set of energetic parameters (Fyhn et al., 2001; Nilsson, 2002; Wiersma and Tinbergen, 2003; Tieleman et al., 2008) and the link of those to oxidative stress of the same individuals during reproduction is yet to be explored.

I chose the zebra finch species (*Taeniopygia guttata*), as the model system for the **Experiment II**. The zebra finch is a common bird model used in many studies investigating physiological parameters including those during temperature regulation and water metabolism (Calder, 1964; Rutkowska et al., 2016; Briga and Verhulst, 2017), energetics (Moe et al. 2009; Deerenberg and Overkamp 1999) and reproduction (Rutkowska et al., 2005; Arct et al., 2010; Sudyka et al., 2016). Even though zebra finches are native to Australia, nowadays they can be found in many laboratories around the world (Zann, 1996). The reason behind this is that zebra finches are easy to maintain and breed under laboratory conditions.

Until now, only few studies have tested heat dissipation limit (HDL) hypothesis in free-living birds. Here, my goal was to test HDL hypothesis under laboratory controlled conditions to control a variety of factors. Laboratory conditions allowed me to employ a powerful repeated experimental design with measurements of oxidative stress several times before and along

reproductive activities on the individual level to disentangle the role of oxidative stress during reproduction.

1.6. General Research Questions

To sum up, the specific questions that I am addressing in this Thesis are:

- What is the link between basal metabolic rate and daily energy expenditure during sustained workload? (Experiment I)
- Does the ability to dissipate heat limit breeding females to maximize their energy expenditure for reproduction? (Experiment II)
- What are the costs in terms of oxidative stress during reproduction? (Experiment I, Experiment II)

CHAPTER II Investigating the influence of avian energetics on oxidative stress during reproduction

2.1. Experiment I; a field study in great tits (*Parus major*)

2.1.1. Summary

Reproductive period in most altricial birds requires high daily workloads from the parents to provide food and warmth. Under such energetically demanding conditions, energy allocation may be subject to trade-offs between different demands. Aerobic metabolism is responsible for the production of reactive oxygen species (ROS), and if not quenched through antioxidants - oxidative stress may occur. Any energy allocation towards high reproductive effort and output over self-maintenance may come at the cost of increased oxidative stress. For this study, I experimentally enlarged broods in great tits (*Parus major*) to increase parental workload and to compare measurements of daily energy expenditure (DEE), basal metabolic rate (BMR) and oxidative stress (uric acid, d-ROM, OXY) during the peak of offspring-rearing period. Firstly, I hypothesized that brood enlargement will increase DEE compared to the control broods. Then, high DEE will be either supported by a high metabolic machinery hence high BMR ("increased intake hypothesis") avoiding oxidative stress or supported by lowered BMR ("compensation hypothesis") reducing investment towards self-maintenance on the cost of increased oxidative stress. As predicted, females raising enlarged broods revealed higher DEE in comparison to control, but BMR remained unchanged. Females raising enlarged broods revealed higher sustained metabolic scope (DEE/BMR) around 3.1 x BMR compared to controls with 2.6 x BMR. Independent of the brood size manipulation, high DEE and metabolic scope came at the cost of decreased non-enzymatic antioxidant capacity and increased

oxidative stress index (d-ROM/OXY), but not related to uric acid and oxidative damage. Brood size manipulation successfully increased DEE and metabolic scope and females invested towards reproductive output to meet the enhanced offspring demands than control females, over self-maintenance. However, the increase in DEE could not fully compensate for the experimental increased demands, enlarged broods had nestlings with lower body mass and shorter wing length and structural size (tarsus length) when compared to control broods. No link between DEE and BMR could be identified. The negative link between metabolic scope and antioxidant capacity comes in the line with the hypothesis that oxidative stress comes as a physiological cost during reproduction. Intensive physical activity in females raising enlarged broods, evident though increased DEE and metabolic scope, due to continuous chick-provisioning may have caused the depletion of the internally stored antioxidants for removing the excess ROS production.

Figure 2. The great tit (*Parus major)* species on a wooden nest box in Niepołomice forest (source: Christina Ninou), a typical nest of the great tit with laid eggs (white with reddish

spots), great tit hatchlings one day after egg-hatching and nestlings just before fledgling (source: Elisavet Zagkle).

2.1.2. Methods and Materials

Study site

This study was conducted on a great tit population (*Parus major*) in breeding season 2018 at a nest box colony in Niepołomice Forest, Poland (50.11 °N, 20.42 °E). Niepołomice forest is characterized as a 60-100 year old woodland with oak (*Quercus sessilis*), scots pine (*Pinus sylvestris*), birch (*Betula verrucosa*) and spruce (*Picea excelsea*) trees (Cichon and Linden, 1990). At the beginning of the breeding season, a total of 275 nest boxes were regularly checked to identify the bird species, to estimate the incubation state, clutch size, and finally observe the hatching date for the great tits (see Figure 2). The average clutch size of the breeding pairs was 11.01 ± 1.4 SD for a total of 74 great tit nests occupied the nest boxes for this season. First laying date was recorded on 13th April and the first hatching date on 6th May.

Figure 3. A 60-200 year old oak woodland forest and a wooden nest box attached to an oak tree.

Individuals and brood size manipulation

To increase the reproductive effort, a brood size manipulation was applied (Burness et al., 2000) on the day after the first hatching was observed for the respective nest (hatching date was recorded as day 0). Two experimental groups, control and enlarged (Figure 4, Table 2), were assigned based on the true hatching date and the intended clutch size, which is commonly used to control for environmental factors (Williams and Vézina, 2001). For enlarging the brood, hatchlings were obtained from donor nests that were not in the interest of this study. Before adding the donor hatchlings to the assigned enlarged nests, the tip of their hind claws was clipped to follow their survival until the last day of the measurements. The adults and the remained hatchlings of the donor nests were not of interest to the current study and they were not accounted for further measurements. Neither clutch size (ANOVA: $F_{1,20} = 0.05$, $p = 0.82$) nor hatching date differed between the experimental groups (ANOVA: $F_{1,20} = 0.04$, $p = 0.83$). Hatching during this season was relatively asynchronous, with most eggs hatching on day 0 but some eggs did not hatch and thus, either one or two hatchlings were added to bring the

brood size back to the intended clutch size for both control and enlarged nests. At the end, the average change in number of chicks between the manipulation and the intended clutch size for control group was 0.1 ± 0.3 (mean ± sd, n = 10) and for enlarged group 3.4 ± 0.5 (mean ± sd, n = 10). Number of chicks on day 15 post-hatching significantly differed between the experimental groups; control (LSE ± SE: 9.3 ± 0.7, n = 10) and enlarged (LSE ± SE: 12.2 ± 0.7, n = 10) (ANOVA; $F_{1,18} = 8.83$, $p = 0.008$).

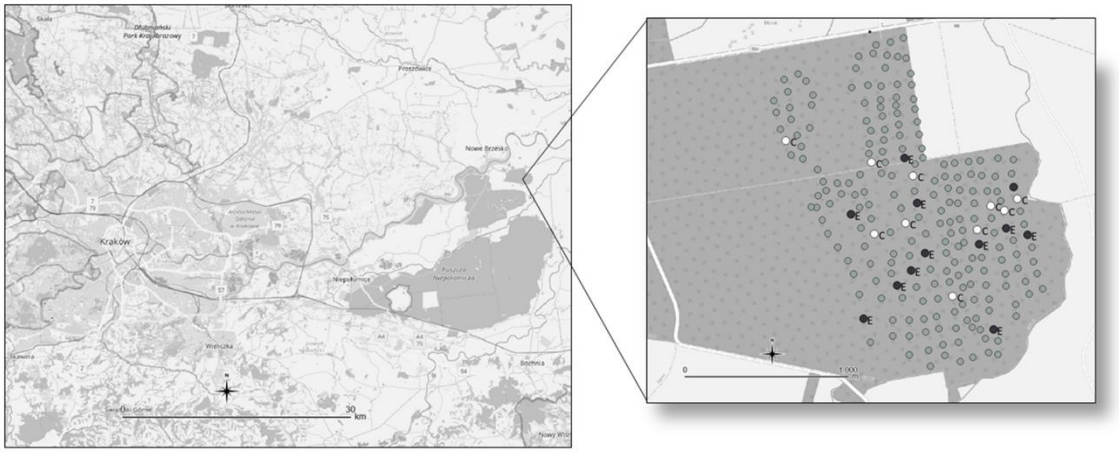

Figure 4. Map of the Niepołomice forest area and the nest box colony (grey points). White points represent the control nest boxes and black points the enlarged manipulated nests of this study.

Standard experimental procedure

At a nestling age of 8 to 12 days the parental investment is at its peak energetic demand due to continuous food provisioning (Drent and Daan, 1980). Thus, adult females were captured on day 9, one hour before the sunset during night-roosting in the nest box. If females were not present in the nest boxes then females were captured using either mist nets or traps (one hour before sunset). After capture, birds were ringed with an aluminium ring having a unique identification number, aged and sexed based on (Svensson, 1992). Body mass was recorded

using an electronic pocket balance ± 0.1 g (KERN 440-45N, Kern & Sohn GmbH, Germany) and morphological measurements such as the wing and the tarsus length using a ruler and a calliper respectively. After the measurements, birds were transferred to perform basal metabolic rate (BMR) measurements (see below for BMR description) during the night from 21:00 to 03:00 at ambient temperature of 25 °C, which is within the thermoneutral zone of this species (Broggi et al., 2010). At the end of the respiratory measurements, birds were injected intraperitoneally with an average of 0.12 ml mixture of isotopes (provided by the Energetics research group, University of Aberdeen, Scotland). Body mass was measured once more and birds were let to rest inside a bird bag for one hour to allow the isotopes to equilibrate within the body. After rest of one hour, a blood sample of 150 µl was obtained from the jugular vein using insulin syringes (0.1 ml; needle 0.3 mm, Becton Dickonson). Blood was collected separately for doubly-labelled water (DLW) assessment in 2 ^{x} 50 µl capillaries (see below for further description of the DLW method) and for oxidative stress biomarkers in heparinized Eppendorfs (see below description). Birds were released after collecting the blood samples. The next day one hour before sunrise nest boxes were re-visited to capture females either directly from the nest box, or using a mist-net or trap, to obtain the final blood sample from the wing vein. I was not able to re-capture all females or some blood samples were not enough for making all the measurements, resulting in a reduced sample size (see sample size for each measurement on Table 2). On day 14, I visited nests for measuring the nestlings; they were ringed with an aluminium ring, and recorded for body mass, wing and tarsus length (Figure 5). The nest boxes were revisited for the last time on day 35 and any nestlings that did not fledge were recorded to determine fledgling success.

Table 2. The different measurements along the experiment and the sample size (n) from the day the brood size manipulation took part (day 2). BMR; Basal metabolic rate, DEE; Daily Energy Expenditure, OS; Oxidative Status

Brood size manipulation group	Day 1	Day 9 (BMR)	Day 10 (DEE)	Day 10 (OS)			Day 14 (nestlings)
				d-ROMs	OXY	UA	
Control	10	10	9	9	8	7	10
Enlarged	12	12	9	8	8	7	11

Figure 5. A nest box full of nestlings on day 14 and a nestling on day 14 during the measurements where at this stage nestlings reach a body mass very similar to adults.

Basal metabolic rate of females during nestling feeding period

Basal metabolic rate (BMR) represents the amount of energy that an organism requires for maintaining homeostasis and it is typically measured when an animal is resting, non-reproducing and post-absorptive under thermoneutral conditions (Speakman, 2000; Speakman et al., 2003). Generally, BMR in great tits is higher during egg-laying phase than the nestling-feeding period (Nilsson and Råberg, 2001) and thus, we may assume that BMR in our study

which was performed almost 20 days after egg-laying phase represents the minimum energetic costs for living. The measurements of BMR of this study took part on day 9 post-hatching, during night-time while resting and ambient temperature of 25 °C, which is within the thermoneutral zone of the great tit species (Broggi et al., 2010). Respiratory measurements to estimate BMR were performed using a nine-channel respirometric system; eight channels for measurements and one baseline channel (Sable Systems, Inc., Las Vegas, NV, USA). Great tit females were placed in sealed individual chambers inside a dark climatic "room" with controlled ambient temperature of 25 °C. Individual chambers were built from typical commercial glass containers of 1100 ml volume and painted externally with black colour to maintain dark conditions. Inside, a tight metal cage was placed for restraining birds from flight movements and attached to the cage a wooden stick for allowing birds to perch during the measurements. An inlet tube was inserted inside the chamber around 2 cm above the bottom and outlet at the top which allowed a good air flow inside the chamber. The bottom of the individual container was filled with 50 ml of white mineral oil (AnVit, Poland) to collect faeces. A fresh sample of air in standard pressure and room temperature was dried with silica gel driers and divided into 9 streams; eight was pumped into chambers with eight mass flow system pumps 2LPM (Sable System International, USA), and one for baseline reference. Air flow was set at 500 ml min^{-1} through chambers and regulated separately for each chamber. Samples from chambers were synchronized sequentially through Intelligent Multiplexer MUX (Sable System International, USA). Sub-sample of air stream was first analysed for water content and then was pre-dried using permeable-membrane dryer and then dried with magnesium perchlorate (Anhydrone, J.T. Baker, USA) columns before passing the CO_2 and O_2 analyser. Gas concentrations were recorded every one second through a Field Metabolic System and were analysed and downloaded through ExpeData software (Sable System International, USA). Representative O_2 concentration value was calculated for each bird from

the values recorded at the last 20 seconds just before switching the channels similar to Sadowska et al., (2015). The first seven cycles of measurements were not accounted in order to obtain the minimum value of metabolic rate (used by us as BMR) to assure that birds were under a post-absorptive state. Finally, BMR was converted to obtain the energy equivalents in Watts according to the following formula (Lighton, 2008) where RQ (respiratory quotient) was calculated as RQ = VCO2/VO2:

$$BMR\,[W] = BMR[ml\ O_2 min^{-1}] \times (16 + 5.164 \times RQ)/\,60$$

Daily energy expenditure during nestling-rearing

Daily energy expenditure (DEE) of the females was estimated as the energy expenditure within 24h-period during the chick-rearing period, which is considered as sustained workload period (Drent and Daan, 1980; Peterson et al., 1990) using the doubly-labelled water (DLW) method (Speakman, 1997). This technique and DEE estimation was performed with the help of collaborators with Prof Dr John R. Speakman and Dr Catherine Hambly at the Energetics Research Group at the University of Aberdeen in Scotland. This method has been previously validated by comparison to indirect calorimetry in numerous animals (e.g. Speakman, 2000). DLW technique uses turnover rates of isotopes introduced into body water to calculate production of CO_2 and, in turn, energy expenditure (Speakman, 1997). In this study, after the respiratory measurements, birds were injected intraperitoneally with an average of 0.12 ml mixture of isotopes, a known mass of enriched oxygen (^{18}O) and hydrogen (^{2}H) (665460 ppm ^{18}O, 328410 ppm ^{2}H; provided by the Energetics research group, University of Aberdeen, Scotland). Birds were weighed and left inside a bird bag for one hour (for equilibrium) before taking the initial blood samples (around 150 µl) from the jugular vein using insulin syringes (0.1 ml; needle 0.3 mm, Becton Dickonson). Blood sample was then collected in 2 x 50 µl

heparinized glass capillaries (blood gas capillary tubes, Vitrex company), and they were immediately fire sealed and stored with some cotton as a protection in plastic tubes of 15 ml. The remaining blood sample of 50 µl was collected in heparinized Eppendorfs for estimating oxidative stress markers (see below description). Birds were released, and after a 24h-period from the initial blood sample, they were recaptured to obtain the final blood samples, this time from the wing vein (total 100 µl separated in 2 x 50 µl heparinized glass capillaries; Vitrex company). To estimate the background isotope enrichments of ^{2}H and ^{18}O, three birds that were not included in the experiment (and thus not injected with DLW) were captured and bleed. After fieldwork all samples were sent by regular post to the Energetics Research Group. Analysis of the isotopic enrichment of blood was performed blind, using a Liquid Isotope Water Analyser (Los Gatos Research, USA) (Berman et al., 2012). Initially the blood encapsulated in capillaries was vacuum distilled (Nagy, 1983), and the resulting distillate was used for analysis. Samples were run alongside five lab standards for each isotope and International standards to correct delta values to ppm. A single-pool model was used to calculate rates of CO_2 production (Speakman, 1993).

Oxidative status biomarkers

From the total blood sample (150 µl), around 50 µl was collected in heparinized Eppendorfs (from the insulin syringes, see above) for further analysis of the oxidative stress biomarkers. Once blood sample was collected it was immediately centrifuged for 5 minutes at 3340 g to separate plasma (Centrifuge MPW-56, MPW Med. instruments). Plasma samples were separated with a pipette and then stored at -20 °C until the end of the fieldwork (around two weeks). Afterwards, plasma samples were transferred to -80 °C until further analyses. I estimated the oxidative status of the birds by measuring early oxidative damage, non-enzymatic antioxidant capacity and uric acid in plasma using the d-ROMs, the oxy-adsorbent (OXY) and

uricase colorimetric tests respectively (Diacron International, Grosseto, Italy). Each sample from one individual was assayed on the same plate (a 96-well microplate; A:H x 1:12) to avoid any differences within individual due to the plate variation, while samples from either enlarged or control groups were randomly distributed during the analysis on the plate. All analyses of the plates for the colorimetric assays were run using an absorbance reader (Sunrise, Tecan's Magellan, Tecan Trading, Switzerland). For some individuals, plasma was not enough for carrying out all analysis (d-ROMs, OXY and uric acid). As a result, I estimated d-ROMs from eight control females and seven enlarged manipulated females, OXY from eight females for both groups, uric acid from seven females for both groups.

The d-ROMs test measures reactive oxygen metabolites (ROMs) that include mostly hydroperoxides. Hydroperoxides are early peroxidation products when ROS interact with many different biological macromolecules. In plasma, hydroperoxides are produced during lipid oxidation events and are correlated with levels of circulating isoprostanes, an end product of lipid peroxidation events. Because hydroperoxides are precursors of lipid peroxidation products such as malondialdehyde or isoprostanes, they are more likely to reflect a whole-animal response to changes in ROS as they occur earlier in the oxidative cascade than those end products (Ito et al., 2017). In this test, the ROMs of the plasma, in the presence of iron, generate the alkoxyl and alkylperoxyl radicals that are highly reactive (Sudyka et al., 2016). When these compounds oxidize an aromatic amine, contained in the chromogen, they produce a complex whose colour intensity is directly proportional to the plasma concentration of ROMs (Costantini and Dell'Omo, 2006a). Initially, 4 µl of plasma was diluted with 200 µl of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and N,N-diethyl-p-phenylenediamine as chromogen and then incubated for 75 min at 37 °C with continuous mild shaking (75 rpm) (Markó et al., 2011). After reacting with an alkyl-substituted aromatic amine solubilized in the chromogen, the metabolites produce a complex whose colour intensity is

directly proportional to their concentration. After incubation, the absorbance was read at the spectrophotometer at 505 nm. The concentration of ROMs was calculated by comparison with a standard curve obtained by measuring the absorbance of a standard solution (Sudyka et al., 2016). The results are expressed in units as mmol/H_2O_2 equivalents.

The total non-enzymatic antioxidant capacity in plasma was quantified as the ability of the plasmatic antioxidant barrier to cope with the oxidant action of hypochlorous acid (HClO) which is an endogenous oxidant in biological systems (Markó et al., 2011; Sudyka et al., 2016). The plasma (2 μl) was diluted with distilled water in 1:100. A 200 μl aliquot of HClO was added to the diluted plasma and further incubated for 10 min at 37 °C with mild shaking at 75 rpm. After incubation, 2 μl of the chromogen solution (similarly to the one for the d-ROMs test), an alkyl-substituted aromatic amin, was added and oxidatzed by the HClO residual transforming the sample into a pink derivative. The intensity of the colour complex is inversely related to antioxidant capacity (the more "stuff" the less colour is produced) (Costantini and Dell'Omo, 2006a). The absorbance was read at the spectrophotometer at 490 nm. The results of the OXY assay are expressed as mmol of HClO neutralized of the sample according to the following formula:

$$\frac{(Absorbance\ reagent\ blank - \ absorbance\ sample)}{(Absorbance\ reagent\ blank - absorbance\ calibrator)}\ x\ [Calibrator]$$

The plasma concentration of uric acid was measured by the endpoint uric acid assay kit (Diacron International, Grosseto, Italy), similarly to the protocol from Zagkle et al., (2020). In each well of the 96-well microplate, 2.5 μl of plasma (similarly for blank sample and standard) was mixed with 100 μl of reagent, incubated and shaken in 37 ºC for 5 min and measured in 510 nm wavelength. The colorimetric uric acid test uses uricase enzyme to convert uric acid to allantoin and the reaction with hydrogen peroxides allows the spectrophotometer to read the concentration of uric acid. The values of uric acid in the plasma are expressed as mmol/L.

I also estimated oxidative stress index by calculating the ratio of the ROMs to the total non-enzymatic antioxidant capacity in the plasma multiplied by 1000 (ROMs/OXY x 1000), similarly as in (Costantini et al., 2006).

Statistical analysis

I performed general linear models to test for differences in DEE, BMR and oxidative stress biomarkers between females raising control (n = 10) and enlarged (n = 10) broods. Brood size manipulation variable was included as a predictor variable, while body mass (in the morning), the date of the measurements and the number of hatchlings on day 14 were included as covariates to account for the possible variation in the dependent variables (DEE, BMR and oxidative stress biomarkers). Interaction between the brood size manipulation variable and body mass in the morning was also included in the models but it was not significant and thus removed. In the case of the model with BMR as a response variable, the number of individual respirometer chamber was also included as a categorical variable to account for possible differences during the measurements between the respirometer chambers. For the relationship between DEE and oxidative stress biomarkers, sample size does not include two individuals from which they were not captured second time to obtain second blood sample for the DLW water method. I performed an analysis of covariance (ANCOVA) to test DEE in relationship to oxidative stress biomarkers and the brood size manipulation. Body mass measured in the morning and the number of hatchlings on day 14 were included as covariates to account for possible variation. Linear mixed effect analysis was performed to study the effect of brood size manipulation on nestling body condition quantified by the body mass, wing and tarsus length. The number of chicks was included as a covariate and the date of measurements to account for possible explanation in the variation of the morphological variables. Identification number of

each nest was included in the model to account for the repeated measurements of several chicks inside one nest.

All statistical analysis was performed by using R Studio software (R version 4.0.3, (R, 2021)). Normality and homoscedasticity was visually inspected from the residuals. All variables verified the assumption of normality and homoscedasticity except of the case of oxy-adsorbent (OXY) variable, which was log-transformed to obtain normal distribution and homoscedasticity. Linear mixed effect models was performed using "lmer" function from the *lme4* package (Bates et al., 2015) and *lmerTest* package (Kuznetsova et al., 2017) to calculate the degrees of freedom and p values. Least square means (LSM) and standard error means (±SE) were calculated using *emmeans* package (Lenth, 2022). I checked possible interactions between the factors and all non-significant interactions ($p > 0.05$) were removed from the models (if not in the interest of the research question). All figures presented are based on raw data unless it is otherwise stated. The sample size in many biological experimental studies may not always reach a sufficient amount (i.e. for logistic reasons as in this study) and thus decreasing considerably the statistical power. Results are presented and the biological effects are interpreted in a language of evidence (Muff et al., 2022) and clarity (Dushoff et al., 2019) rather than in the cut-off binary decision of the p-value<0.05.

2.1.3. Results

a) Energetic parameters

There was no evidence that daily energy expenditure (DEE) is associated with body mass measured in the morning ($F_{1,14} = 1.67$, $p = 0.21$). The data revealed a moderate evidence that brood size manipulation (categorical variable with two groups: control and enlarged) affected DEE ($F_{1,14} = 6.24$, $p = 0.02$; Figure 6a). Females from the enlarged broods revealed a 12.8% higher DEE (n = 9, LSM ± SE: 1.14 ± 0.03 Watts) in comparison to the females from the control broods (n = 9, LSM ± SE: 1.01 ± 0.03 Watts; Figure 6a). On the other hand, there was no evidence that brood size manipulation has an effect on basal metabolic rate (BMR) ($F_{1.13} = 0.42$, $p = 0.52$; Figure 6a). BMR did not differ between the manipulation groups, control (n = 10, LSM ± SE: 0.41 ± 0.01 and enlarged (n = 12, LSM ± SE: 0.39 ± 0.01). Also, BMR was not evidently associated with body mass measured in the morning (post-BMR) ($F_{1,15} = 0.03$, $p = 0.85$) and the individual respirometer chamber ($F_{5,15} = 2.29$, $p = 0.09$). Sustained metabolic scope (SusMS = DEE/BMR) was found to differ between the brood size groups ($F_{1,15} = 6.17$, $p = 0.02$), with females from the enlarged broods revealing in average 3.1 × BMR (s.d. = 0.3), while females from the control group 2.6 × BMR (s.d. = 0.4) (Figure 6b). Regarding the link between DEE and BMR, the data did not show any evidence about the direction of any association ($F_{1,13} = 0.00$, $p = 0.99$; Figure 6c) regardless of the brood manipulation group ($F_{1,13} = 1.32$, $p = 0.27$).

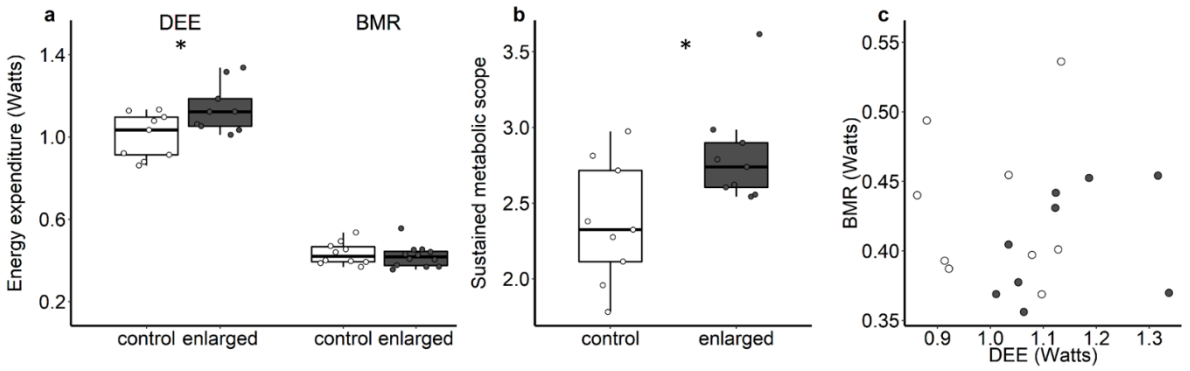

Figure 6. Energy expenditure and brood size manipulation groups in female great tits (*Parus major*); a) over a 24-hour period (daily energy expenditure: DEE), and measured during resting and thermoneutral conditions (basal metabolic rate: BMR), b) sustained metabolic scope, and c) relation of BMR and DEE of females from the enlarged group (filled symbols) and females from the control group (open symbols). Boxplots show the median, the range and the inter-quartiles, while the circle points the individual values. Graphs are jittered to prevent over-plotting between the individual points for better visualization of the distribution. Asterisks indicate statistical significance between the brood size groups.

b) Oxidative status

There is no evidence that uric acid differs in females from the control and the enlarged broods ($F_{1,10} = 0.00$, $p = 0.93$; Figure 7a). Similarly, there is no evidence that non-enzymatic antioxidant capacity (OXY), after log-transformation, is different between the two experimental groups of females ($F_{1,12} = 2.64$, $p = 0.13$; Figure 7b) and neither oxidative damage ($F_{1,12} = 0.87$, $p = 0.36$; Figure 7c). Oxidative stress index, estimated as the ratio between oxidative damage and antioxidant capacity multiplied by 1000 (Costantini et al., 2006), was higher in females from the enlarged broods than the females from the control broods ($F_{1,12} = 5.50$, $p = 0.03$; Figure 7d). Neither body mass nor date of the measurements explained variation in any of the oxidative biomarkers ($p > 0.01$).

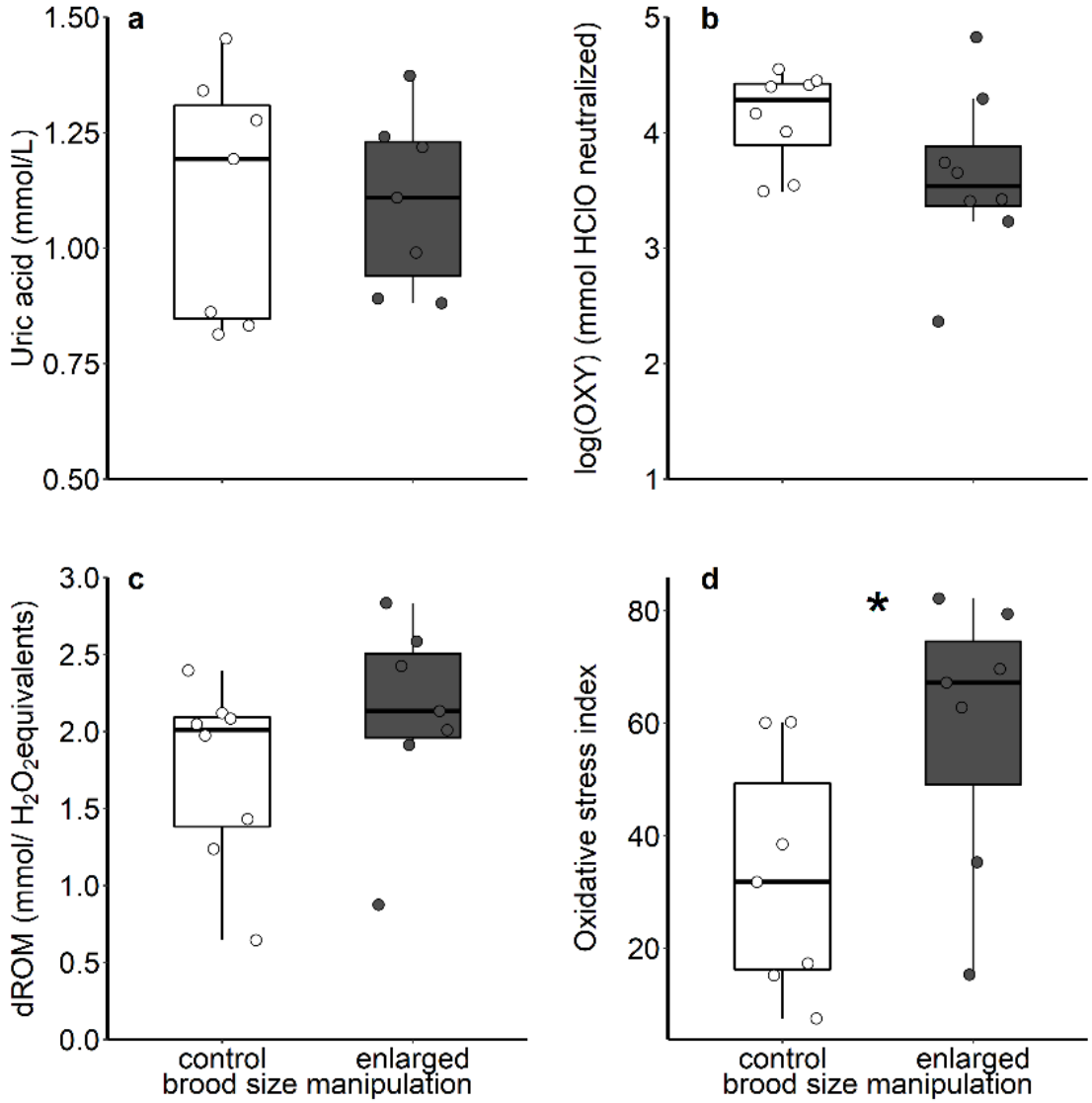

Figure 7. a) Uric acid (mmol/L), b) log transformed OXY (mmol HClO neutralized), a metric for non-enzymatic antioxidant capacity, c) dROM (mmol/H_2O_2 equivalents), a metric of early oxidative damage and d) oxidative stress index, calculated as the ratio of the dROM to OXY multiplied by 1000 in plasma concentration of the great tit females (*Parus major*) between the control (white colour and open symbols) and the enlarged broods (dark grey colour and filled symbols). Boxplots show the median, the range and the inter-quartiles, while the different circle points represent individual values. The points are jittered to prevent over-plotting between the individual points for better visualization of the distribution. Asterisk indicates statistical significance for oxidative stress index ($F_{1,12} = 5.50$, $p = 0.03$).

c) Link between sustained metabolic scope and oxidative status

There was no evidence that uric acid was related to sustained metabolic scope (SusMS) ($F_{1,11} = 0.15$, $p = 0.71$) and neither to the brood size manipulation ($F_{1,7} = 0.02$, $p = 0.87$; Figure 8a). Non-enzymatic antioxidant capacity was negatively related to SusMS ($F_{1,9} = 6.83$, $p = 0.03$) and the brood size manipulation factor (a trend; $F_{1,9} = 4.33$, $p = 0.06$; Figure 8 b). There was no evidence that oxidative damage in females is related to SusMS ($F_{1,10} = 0.00$, $p = 0.99$) and neither with the brood size manipulation ($F_{1,10} = 0.75$, $p = 0.40$; Figure 8 c). Oxidative stress index was positively related to SusMS ($F_{1,9} = 8.9$, $p = 0.01$) and the brood size manipulation effect ($F_{1,9} = 8.34$, $p = 0.01$; Figure 8 d). Interaction of metabolic scope and brood size manipulation was not statistical clear in neither of the aforementioned models.

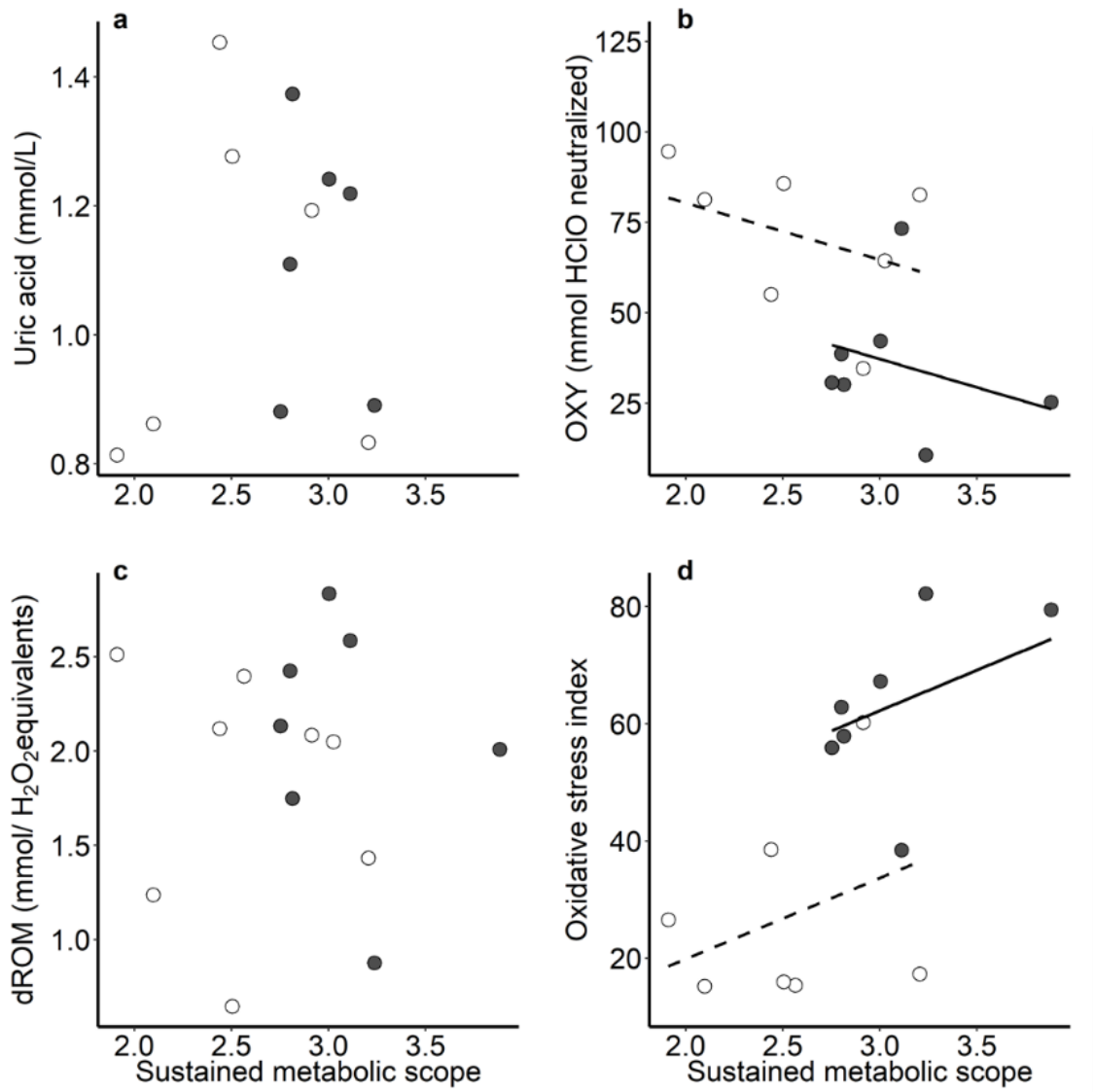

Figure 8. Markers for oxidative stress plotted over SusMS. A) Uric acid (mmol/L), b) OXY (mmol HClO neutralized), a metric for non-enzymatic antioxidant capacity, c) dROM (mmol/H_2O_2 equivalents), a metric of oxidative damage and d) oxidative stress index, calculated as the ratio of the oxidative damage to the total non-enzymatic antioxidant capacity multiplied by 1000 (Costantini et al., 2011) in plasma concentration of the great tit females (*Parus major*) in relation to sustained metabolic scope (=DEE/BMR) of the females raising control (open symbols) and enlarged broods (filled symbols).

d) Link between daily energy expenditure (DEE) and oxidative status

There was only a weak evidence that uric acid is positively associated to DEE ($F_{1,11} = 3.58$, $p = 0.08$; Figure 9) and brood size manipulation ($F_{1,11} = 2.98$, $p = 0.11$). The data revealed a

moderate evidence that non-enzymatic antioxidant capacity is negatively related to DEE (slope = -117.95 ± 71.38, R^2 = 0.46, $F_{1,9}$ = 5.08, p = 0.05; Figure 9) and depends on the brood size manipulation ($F_{1,9}$ = 6.58, p = 0.03), with females raising enlarged brood having lower antioxidant capacity than the control ones. Females with increased DEE revealed with lower non-enzymatic antioxidant capacity, independent of the brood size. There was no evidence that oxidative damage is associated to DEE ($F_{1,11}$ = 2.12, p = 0.17; Figure 9) nor to the brood size manipulation ($F_{1,11}$ = 0.12, p = 0.73). There was moderate evidence that oxidative stress index was positively related to DEE ($F_{1,9}$ = 7.16, p = 0.02; Figure 9) and differed between the brood size manipulation groups ($F_{1,9}$ = 11.04, p = 0.008). Females raising enlarged broods revealed with higher oxidative stress index compared to the females raising control broods.

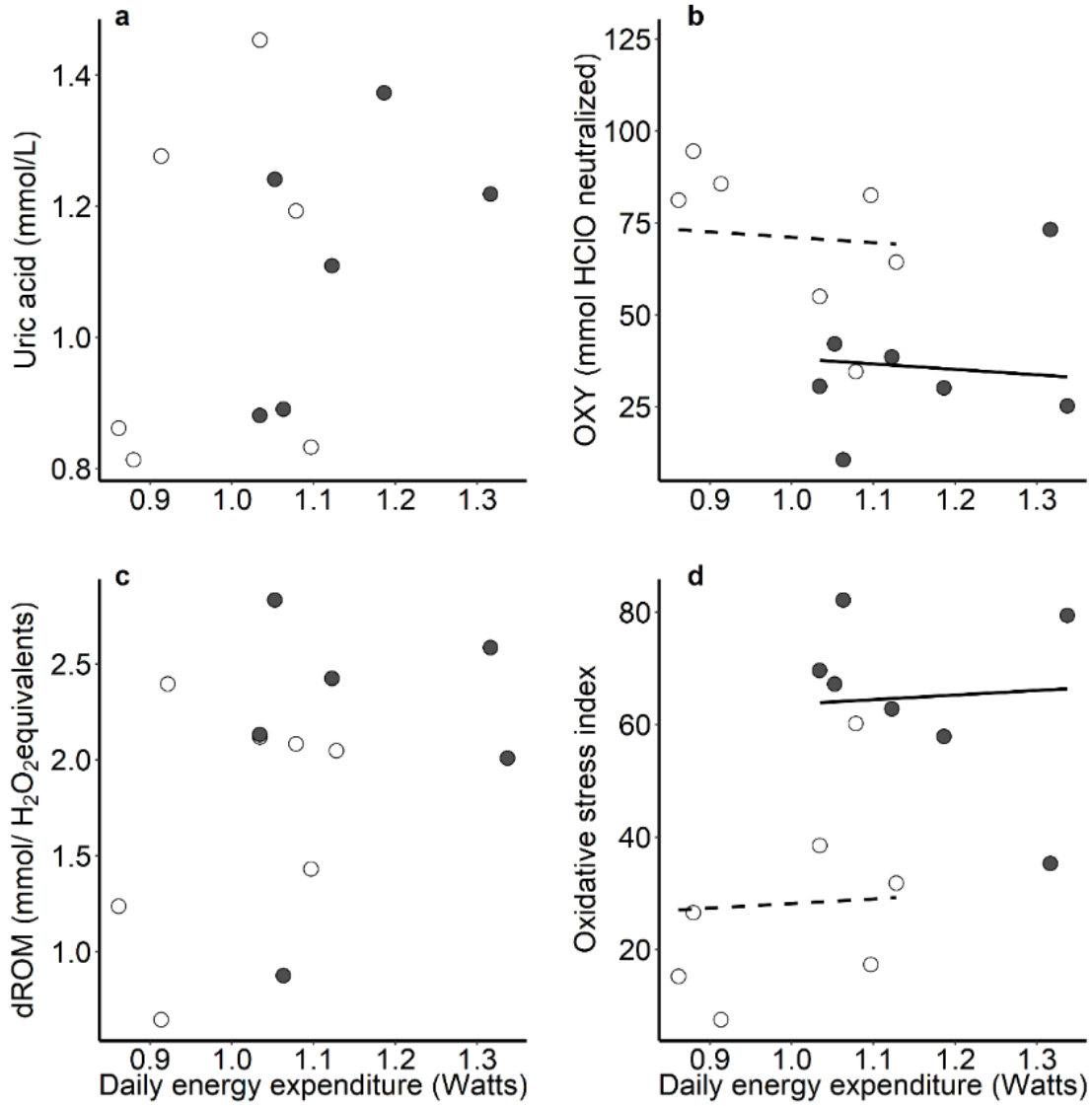

Figure 9. Markers for oxidative stress plotted over DEE. a) Uric acid (mmol/L), b) OXY (mmol HClO neutralized), a metric for non-enzymatic antioxidant capacity, c) dROM (mmol/H_2O_2 equivalents), a metric of early oxidative damage and d) oxidative stress index, calculated as the ratio of the dROM to OXY multiplied by 1000 in plasma concentration of the great tit females (*Parus major*) in relation to DEE of the females raising control (open symbols) and enlarged broods (filled symbols).

e) Link between basal metabolic rate (BMR) and oxidative status

There was no evidence that uric acid is related to BMR ($F_{1,7}$ = 3.16, p = 0.11) and brood size manipulation ($F_{1,7}$ = 0.45, p = 0.52; Figure 10a). Non-enzymatic antioxidant capacity was also

not related to BMR ($F_{1,9}$ = 1.06, p = 0.32) and the brood size manipulation factor ($F_{1,9}$ = 0.43, p = 0.52; Figure 10b) Oxidative damage was neither related to BMR ($F_{1,9}$ = 0.85, p = 0.38) nor to the brood size manipulation ($F_{1,9}$ = 1.54, p = 0.24; Figure 10c). There was no evidence that interaction of BMR and brood size manipulation has an effect in neither of the aforementioned models (but it was not removed from the models since it was in the interest of the research question). Oxidative stress index was not related to BMR ($F_{1,11}$ = 2.14, p = 0.17) but differed between the brood size manipulation groups ($F_{1,11}$ = 6.21, p = 0.03). A moderate evidence was found that interaction between BMR and brood size manipulation group has an effect in oxidative stress index ($F_{1,11}$ = 5.35, p = 0.04). The slope was positive for the control group, (estimate ± SE; 0.25 ± 0.21, t = 1.16, p = 0.27) and for the enlarged group the slope was negative (estimate ± SE; -0.62 ± 0.26, t = -2.34, p = 0.04; Figure 10d).

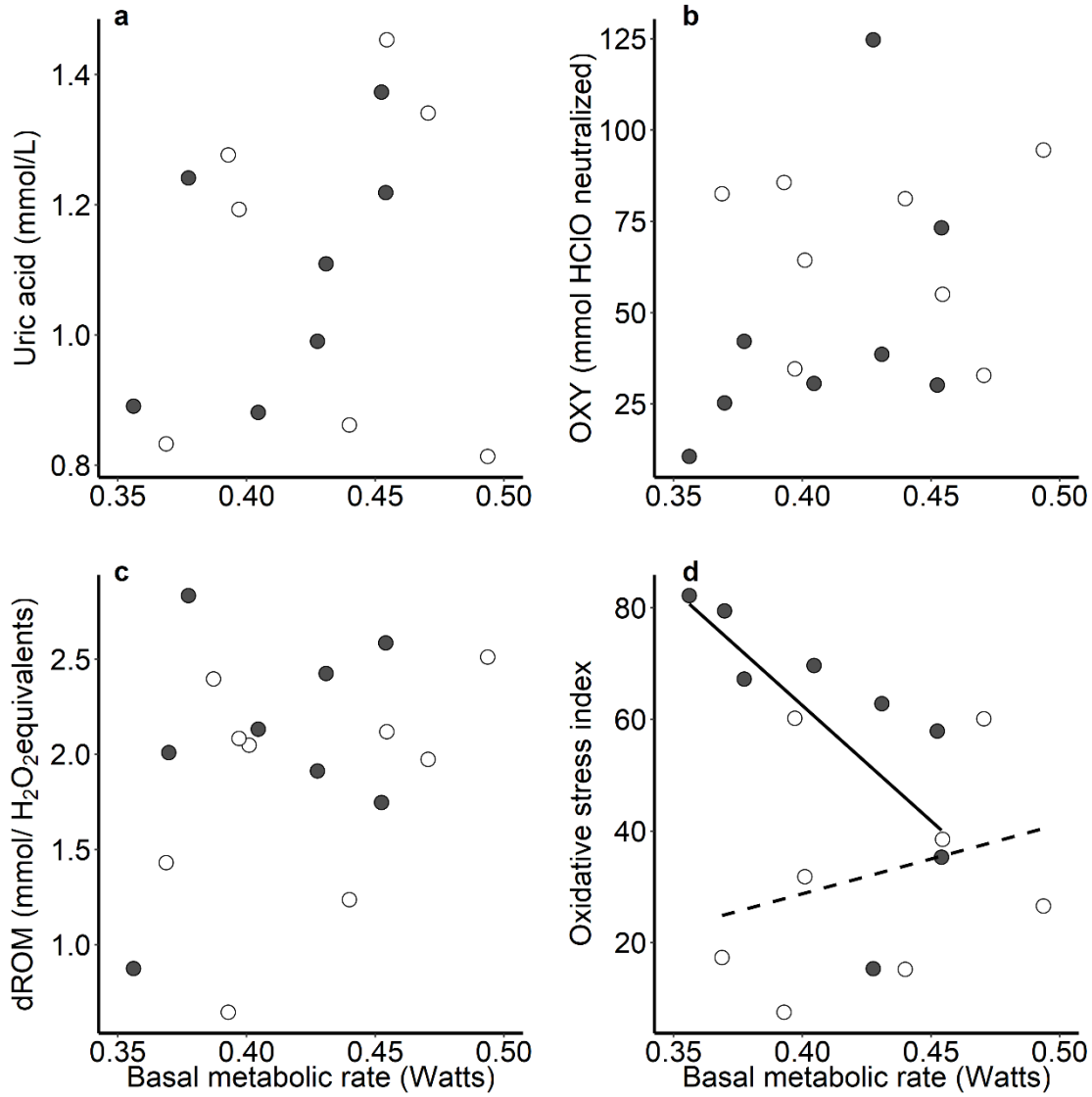

Figure 10. Markers for oxidative stress plotted over BMR. A) Uric acid (mmol/L), b) OXY (mmol HClO neutralized), a metric for non-enzymatic antioxidant capacity, c) dROM (mmol/H_2O_2 equivalents), a metric of oxidative damage and d) oxidative stress index, calculated as the ratio of the dROM to OXY multiplied by 1000 in plasma concentration of the great tit females (*Parus major*) in relation to BMR of the females raising control (white colour) and enlarged broods (dark grey colour).

f) Nestlings

There was no evidence that the clutch mass (sum of the body mass of the nestlings per nest at day 14) differs between the control and enlarged groups ($F_{1,16} = 0.06$, $p = 0.79$). Hatchling

number significantly differed between the groups as result of the experimental manipulation; control (LSE ± SE = 9.3 ± 0.7, n = 10) and enlarged (LSE ± SE = 12.2 ± 0.7, n = 10) (ANOVA; $F_{1,18} = 8.83$, $p = 0.008$). Body mass of the nestlings was significantly lower in enlarged than the control broods (Table 3). Tarsus length was also shorter in enlarged broods than the nestlings in the control broods and similarly, wing length was shorter in nestlings from the enlarged than the control broods (Table 3). Only a weak evidence that body condition, calculated as the residuals from a regression of body mass on tarsus length, is affected by the brood size manipulation; lower body condition in nestlings from the enlarged than the control broods (Table 3). Fledgling success rate, calculated as the number of nestlings fledged per the initial number of nestlings on day 1 of brood size manipulation, did not differ between the control and enlarged broods ($F_{1,20} = 2.14$, $p = 0.15$) and the average fledgling success rate for both groups was 71.4%.

Table 3. Body mass (g), tarsus and wing length (mm), and body condition (body mass/tarsus length residuals) of the nestlings on day 15 post-hatching with regard to the brood size manipulation groups. Values represent least square means (LSM) and standard error (SE). Statistics are extracted from the linear mixed effect model; F-value, degrees of freedom for numerator and denominator (df) and p-value based on Satterthwaite's method approximation. Bold numbers indicate statistical significance.

Variable	Control		Enlarged		Brood size manipulation			Number of nestlings			Date		
	LSM	SE	LSM	SE	*F*	*df*	*p*	*F*	*df*	*p*	*F*	*df*	*p*
Body mass	17.77	0.57	15.58	0.48	7.10	1,14.62	**0.01**	3.13	1,15.44	0.09	1.94	1,14.58	0.18
Tarsus length	19.76	0.15	19.30	0.12	4.74	1,14.50	**0.04**	0.03	1,15.97	0.86	1.90	1,14.21	0.18
Wing length	4.49	0.10	4.15	0.08	5.17	1,14.45	**0.03**	2.83	1,15.62	0.11	2.57	1,14.33	0.13
Body condition	0.89	0.61	-0.76	0.53	3.42	1,13.86	**0.08**	2.23	1,14.21	0.156	0.80	1,13.81	0.38

g) Nestling mass in relation to female daily energy expenditure (DEE)

There is a strong evidence that the relationship between nestling mass on day 14 post-hatch and female daily energy expenditure (DEE) depends on the brood size manipulation ($F_{1,14}$ = 11.16, p = 0.004; Figure 11. Mean nestling mass on day 15 in relation to female DEE (F1,11 = 0.09, p = 0.75) and brood size manipulation (F1,11 = 4.45, p = 0.05). Interaction between DEE and brood size manipulation effect was significant (enlarged; Est. ± se.= -124.37. ± 34.95, t = -3.56, p = 0.001, control; Est. ± se.= 21.31 ± 43.07, t = 0.49 p = 0.63). the slope was positive (Est. ± se.= 21.31 ± 43.07, t = 0.49 p = 0.63) for the control group, while for the enlarged group the slope was negative (Est. ± se.= -124.37. ± 34.95, t = -3.56, p = 0.001).

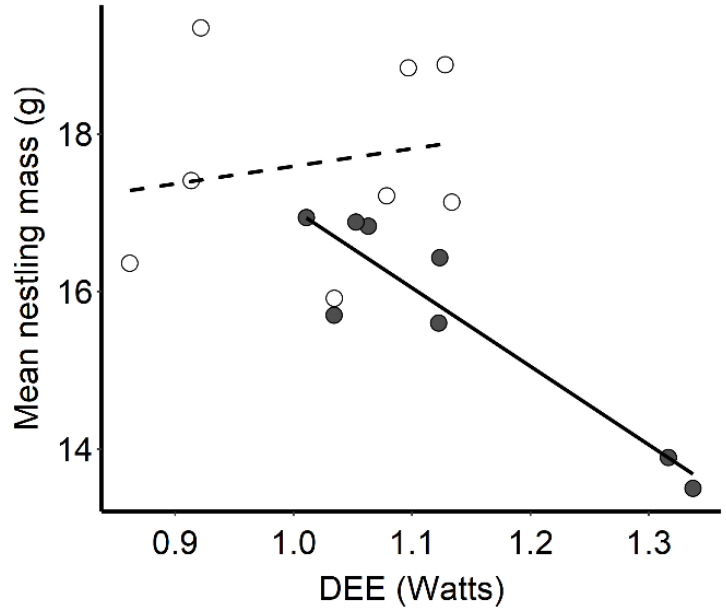

Figure 11. Mean nestling mass on day 15 in relation to female DEE ($F_{1,11}$ = 0.09, p = 0.75) and brood size manipulation ($F_{1,11}$ = 4.45, p = 0.05). Interaction between DEE and brood size manipulation effect was significant (enlarged; Est. ± se.= -124.37. ± 34.95, t = -3.56, p = 0.001, control; Est. ± se.= 21.31 ± 43.07, t = 0.49 p = 0.63).

2.2. Experiment II; a study under laboratory controlled conditions in zebra finches (*Taeniopygia guttata*)

2.2.1. Summary

Often animal life requires hard work but to endure such workload appears challenging. Heat dissipation limit (HDL) hypothesis poses that the capacity to dissipate the excess of body heat during hard work may limit sustained energy use. Experimental facilitations of heat loss rate via feather-clipping in free-living birds seem to support HDL hypothesis, but testing of HDL through laboratory experiments under controlled conditions are not reported. I conducted a two-factorial experimental design to test HDL hypothesis by manipulating the capacity to dissipate heat through exposure of captive zebra finches (*Taeniopygia guttata*) to a cold and warm ambient temperature (factor I: 14 and 25 °C), and through manipulation of the insulating layer of feathers around the brood patch of females (factor II; clipped and unclipped). To simulate foraging costs encountered in the wild and increase foraging effort, I constructed a feeding system that necessitated hovering to access food; this increased energetic costs of reproduction despite *ad libitum* conditions in captivity. Reproductive performance was quantified at the level of both parents during egg-laying period, while maternal reproductive performance during food provisioning period. I followed growth rate of the nestlings to quantify reproductive output. Thermal limitations due to warm temperature already appeared at the beginning of reproduction for both parents with lower egg production compared to the cold. After hatching, females that experienced an increased possibility to dissipate heat through feather clipping revealed higher body mass compared to unclipped females, and clipped females also raised heavier and bigger nestlings. Higher levels for oxidative stress in plasma of females were detected prior to reproduction in warm conditions than in the cold. However, oxidative stress biomarkers were neither affected by temperature nor by feather-clipping during

the reproductive activities. Reduced antioxidant capacity during reproduction may indicate upregulation of the antioxidant defense to neutralize any toxic effects of oxidative imbalance possibly on the costs of female body condition and offspring growth. This study under laboratory controlled conditions corroborates evidence in line with the HDL hypothesis.

Figure 12. A male and a female of the zebra finch (*Taeniopygia guttata*) species from the laboratory colony at the Institute of Environmental Sciences in Kraków, a clutch size formed in the nest, hatchlings just after egg-hatching and nestlings just after fledgling from the nest (source: Elisavet Zagkle).

2.2.2. Methods and Materials

Individuals and temperature set-up

In a two-factorial experimental design, captive zebra finches (*Taeniopygia guttata*) were subjected to two separate manipulations of heat dissipation capacity. Ambient temperature affects the capacity to transfer body heat since heat loss depends on the difference between the body and the ambient temperature (Speakman and Król, 2010), and thus, before reproduction, total 82 pairs of birds were exposed to 14 °C and 25 °C with 39 and 43 pairs, respectively (factor I). Both temperatures are considered below the thermoneutral zone in zebra finches, which ranges from 30 to 38 °C (Calder, 1964; Briga and Verhulst, 2017). During breeding season, zebra finches under natural conditions may experience temperatures ranging from 10 to 30 °C (even in some cases above 36 °C) but most frequently they lay eggs at ambient temperatures of 18 to 20 °C (Griffith et al., 2017). In laboratory conditions, ambient temperature for breeding zebra finches is usually set from 18 to 22 °C to achieve high reproductive success (Rutkowska et al., 2005; Bertrand et al., 2006; Williamson et al., 2008; Arct et al., 2010; Olson et al., 2014). For this reason, I applied two ambient temperatures with a difference of at least ten degrees considering them as cold and warm, respectively. After mating, I documented egg-laying success and hatching success between the two ambient temperatures. About 82% of the 39 pairs (32 out of 39) laid eggs in the cold conditions, while about 51% of the 43 pairs (22 out of 43) laid eggs in warm conditions. From those pairs that laid eggs, a total of 26 out of 32 chicks hatched in cold conditions and 18 out of 22 in the warm (χ^2 = 0.002, p = 0.95). During reproduction, and more specifically on day 6 post-hatching, I applied the second manipulation of heat dissipation via manipulation of the insulating layer of feathers around the brood patch (factor II, feather-clipped versus unclipped females). At the

end, 26 out of 26 pairs successfully raised fully developed chicks until independence at the cold conditions (14 feather-clipped, 12 unclipped), while 14 pairs out of 18 raised fully developed chicks at the warm (7 feather-clipped, 7 unclipped) ($\chi^2 = 6.35$, $p = 0.01$).

In each chamber, the ambient temperature was recorded with six data loggers (thermochrons, DS1921H-F5, iButtonLink, Maxim Integrated products, USA) and the humidity with one additional (hygrochron, DS1923-F5, iButtonLink, Maxim Integrated products, USA) to monitor and maintain the ambient temperature and humidity within established levels. All data loggers were synchronized at the same time to record three times when lights were on ("daytime") and three times when lights off ("nighttime"). Temperature significantly differed between daytime and nighttime for both chambers ($F_{1,7789} = 6.03$, $p < 0.001$). Humidity significantly differed between daytime and nighttime ($F_{1,940} = 46.71$, $p < 0.001$) but it did not differ between the two chambers ($F_{1,940} = 0.001$, $p = 0.97$). The summary of the statistics of temperature and humidity separated for day and night for each chamber is presented below (Table 4).

Table 4. Summary statistics of the temperature (°C) and the humidity (%) for each chamber (see Supplementary Table 1 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Temperature (°C)		**Mean**	**Min**	**Max**	**SD**	**N recordings**
Warm	day	25.2	24	26	0.43	2072
	night	24.7	23.5	26	0.49	2093
Cold	day	14.9	13.3	17.4	0.98	2071
	night	14.6	12.8	14.7	0.95	2093
Humidity (%)		**Mea**	**Min**	**Max**	**SD**	**N recordings**
Warm	day	60.5	52.1	63.5	1.16	2072
	night	61.5	58.8	63.5	0.95	2093
Cold	day	60.2	52.6	66.3	2.58	2071
	Night	61.8	50.8	73.6	4.88	2093

Acclimation period

The colony of zebra finches is usually habituated in a common outdoor aviary at the Institute of Environmental Sciences, Jagiellonian University, Krakow. Thus, at the beginning of the experiment the experimental birds were moved and separated into the two indoor climatic chambers. Birds were initially housed in individual cages (70 × 70 × 45 cm; L × W × H) with two birds of the same sex in each cage and visually separated from other birds. I let the birds to acclimatize for two weeks at the new laboratory conditions with a photoperiod 13:11 (L:D) with lights on at 7 AM and lights off at 8 PM.

High foraging cost feeding system

After two weeks of acclimation, I constructed a feeding system for every cage to increase energetic costs of foraging under *ad libitum* conditions in captivity, similar to a previous technique of (Koetsier and Verhulst, 2011). Such high foraging costs feeders may increase foraging effort by increasing time for foraging and the flights for obtaining food as it was previously shown for zebra finches (Koetsier and Verhulst, 2011; Yap et al., 2017), simulating foraging costs in field conditions. Female zebra finches revealed to have increased flight muscle mass, lung mass and heart mass in response to high foraging cost feeders compared to females exposed to regular feeders (although no differences were detected in males, Yap et al. 2017). Therefore, I constructed a similar feeding system using a transparent plastic feeder and opened one hole fitted with a tube. The feeder was mounted 40 cm from the floor of the cage and initially a wooden stick of 15 cm was attached for perching. After four days, I gradually shortened the wooden stick by half size and birds had access to food while perching from a wood stick of 7 cm. Birds continued to have access to food by perching (wood stick of 7 cm) for a total of nine days and afterwards I removed the perch to start training the birds to fly

towards the foraging hole. Birds obtained the seeds either by constant flights around the feeder or by making repeated hops and jumps from the floor to the feeder hole (around 40 cm). Both flights or hops ended with the bird hovering until they successfully obtained seed from the container (see below Figure 13). I also placed a container below the feeder for the spilled seeds to avoid obtaining seeds from the floor and the container was evacuated every second day.

Figure 13. Female zebra finch (*Taeniopygia guttata*) hovering towards the "high foraging cost feeder" for obtaining seeds. (see Supplementary Figure 2 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

During the acclimation to the new feeding system, I measured body mass in the morning just after lights on and food intake in seed mass (g) over a 24-hour period a) when birds had access to food by perching, b) hovering just after 8 days when perch was removed and c) after 12 days. I performed linear mixed effect model analysis to test differences in body mass between the three measurements (perch, hover after 8 and 12 days). Identification number of each bird

was included as a random effect to account for the repeated measures. I analysed a subset of the experimental birds (n =15 females) exposed to cold conditions but all experimental birds had access to the feeding system. Body mass of the birds was significantly lower when birds had access to seeds while hovering after eight days of removing the perches compared to when birds had access to seeds while perching (Figure 14). However, body mass of the birds, when measured twelve days after removing the perches, returned back to normal values as to the initial body mass when birds had access to food while perching. There was also a sharp decrease in food intake in seeds (g) over a 24-hour period after 8 days of removing the perches compared to the initial food intake in seeds where birds had regular access to food while perching (Figure 14). However, seed mass (g) over 24 hour period did not increase evidently when birds had access to the seeds while hovering measured when perch was removed after 12 days (Figure 14). These results indicate that body mass of the birds recovered their body mass after 12 days of removal of perches subsequent to the short-term drop. Perhaps, body mass increased without an evident increase in seed mass after 12 days of hovering is related to increased organ masses (i.e. flight muscle mass, lung mass, heart mass) as shown in previous study (Yap et al., 2017). Birds to self-maintain were necessitated to obtain seeds while flying and hovering or making repeated jumps from the floor to the feeder, behaviours that are considered energetically costly for birds in the field (Yap et al., 2017), thus stimulating foraging costs as in natural conditions.

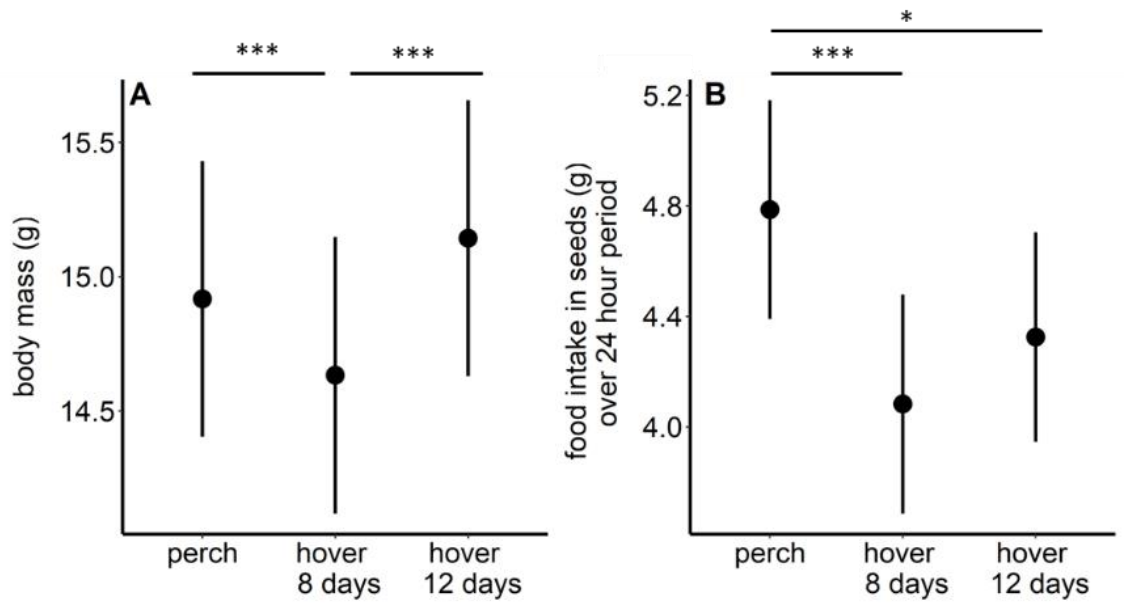

Figure 14. (A) Body mass (g) of female zebra finches and (B) seed mass (g) as calculated from a 24-hour period (in one cage for two birds, thus divided) when measured in the morning just lights on when had access to seeds while perching, while hovering after the perch was removed 8 days before and 12 days before. Points represent least square means and error bars 95% confidence intervals. Asterisks indicate statistical significance after post-hoc analysis (Tukey), with $p < 0.001$ (***) and $p < 0.05$ (*). (see Supplementary Figure 3 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

The training procedure to the high foraging cost feeder lasted for two months. When all birds were trained to the new feeding system, I let birds to acclimatize for three weeks at the ambient temperatures of 14 and 25 °C before the mating. During the whole experiment and reproduction, all birds had unlimited access to food (a mixture of different millet species; Megan, Poland), but as outlined I induced their physical exercise for foraging to simulate natural conditions.

Experimental procedure of mating

After the training to the new feeding system and three additional weeks of acclimation period, I randomly mated the birds with unrelated partners and randomly separated the pairs in four

different blocks. Each block was assigned for mating with four days of interval. On the mating day, each individual cage was equipped with an internal carton-made nest box and nest-material (wood wool and shredded toilet paper) which was replaced every day until birds stopped using it. After the mating, nest boxes were checked every morning between 10 and 11 AM in order to inspect the nest-building stage, the egg laying date (females lay one egg per day early in the morning) and the start of incubation. During the egg-laying period, I marked all eggs alphabetically with a marker, in the sequence they were laid, and each was weighed them with an electronic balance (Kern MM 60 2N ± 0.01, Kern & Sohn GmbH, Germany). When incubation started, birds were supplied three times per week with a small spoon of a mix of chopped hard boiled eggs (with the shell), grated carrot and supplementary vitamins. Birds also had unlimited access to water and to a piece of cuttlebone for the entire period.

Adult measurements

Just before mating, I caught all birds early in the morning to measure initial body mass using an electronic balance (± 0.1 g; KERN 440-45N, Kern & Sohn, GmbH, Germany). When first eggs hatched, which was assigned as day 0, I also measured female body mass just after lights on, and repeated the body mass measurements on day 4, 6, 8, 10, 12, 16 and day 35. At the same time that each female was captured from the cage, chicks from each nest were also captured to measure body mass to follow growth rate (more details on nestling measurements see below). On day 4, I removed the male from the cage to increase the physical activity of the female, assuring that the reproductive effort is only operated from a single parent. On day 6, I applied the feather clip manipulation by trimming the feathers around the brood patch and those covering the pectoral muscles. I established two manipulation groups: feather-clipped and unclipped females, which were handled and measured in the same way but not feather-clipped (sham-manipulation). On day 12, just before lights off, females were caught from inside the

nest-box for performing respiratory measurements to estimate thermal conductance of the females (for more details see below the paragraph on thermal conductance). Last, females were blood-sampled three times during the experiment: a) just before mating (after taking initial body mass) b) on day 13 just after respiratory measurements and c) at the end of the experiment, on day 35, when chicks reached full independence. For a visual summary of the different adult measurements along the experiment see below Figure 15.

Nestlings

During the expected hatching date, I monitored the nests every day between 10 and 11 AM to inspect for possible newly fresh hatchlings. I distinguished day of hatching based on the presence of eggshells' leftovers and the appearance of the hatchlings, since fresh hatchlings are reddish and wet. The true hatching date was recorded as day 0 and the true age of each hatchling was assigned accordingly. Each hatchling was immediately measured with an electronic balance (Kern MM 60 2N ± 0.01, Kern & Sohn GmbH, Germany), and marked by nail clipping of their claw and quickly they were returned back to the nest. Body mass of the nestlings was measured several times during mornings (from 7 to 10 AM) until they reached adult size to capture growth rate (day 0, 4, 6, 8, 10, 12, 16 and day 35). On day 16, just before fledgling, chicks were ringed with an individually numbered aluminium ring and measured tarsus and wing length. On day 35 when offspring almost reach juvenile size I measured for the last time their body mass, tarsus and wing length to estimate their final juvenile size. For a visual summary of the different nestling measurements along the experiment see below Figure 15.

Food intake

Food intake was estimated by measuring the initial seed mass and after 48-hour period the final seed mass. I also took account the spilled seeds in the container that were not obtained and consumed from the birds. I measured food intake over a 48-hour period during the peak of food provisioning and reproductive performance on day 4 to 6, day 6 to 8, day 8 to10 and day 10 to 12. For a visual summary of the different measurements of seed mass to estimate food intake during chick rearing period see below Figure 15.

Oxidative status biomarkers

Females were bleed three times during this study: a) before mating (blood sample 1), b) on day 13, just after respiratory measurements (blood sample 2) and c) at the end of the experiment, on day 35 (blood sample 3), when chicks reached full independence (Figure 15). Blood sampling always took part early in the morning just after lights on. After a brachial vein puncture, I collected blood sample of 75 µl in capillaries and stored in heparinized Eppendorfs 100 µl. Blood sample was immediately centrifuged for 10 minutes at 3340 g to separate plasma (Centrifuge MPW-56, MPW Med. instruments). Plasma sample was stored at -80 °C until further analyses which took place within one month after the end of the experiment. I estimated early oxidative damage by performing d-ROMs test, non-enzymatic antioxidant capacity (OXY), uric acid and oxidative stress index (d-ROMS/OXY x 1000) in plasma concentration following the steps as described at the Methods and Materials for the Experiment I (See section Oxidative status biomarkers, page 44).

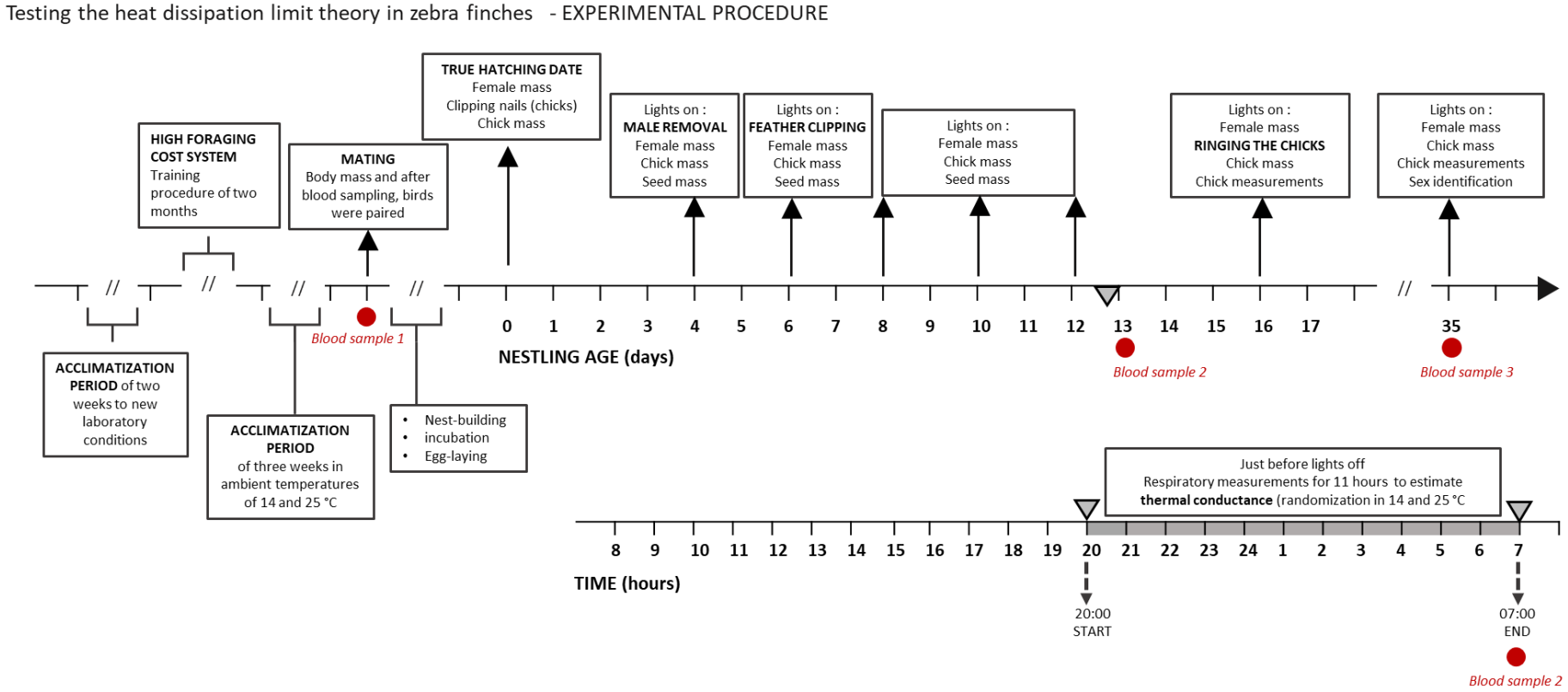

Figure 15. A summary of the timeline and the different steps along the experiment. (modified from Supplementary Figure 1 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Thermal conductance

Thermal conductance measures the ease with which the heat enters or leaves an organism's body. Thermal conductance is rather complex to estimate directly since heat exchange between the organism and the environment occurs through radiation, conduction, convection, and evaporation and each of those may depend on the behaviour, physiology and morphology of the organism or/and the environmental conditions (Naya et al., 2013). Thermal conductance in this study was estimated from the slope of the regression of O_2 consumption and the ambient temperature below thermoneutral zone (Lovegrove, 2003). This the most common and readily available estimate of thermal conductance in the literature (Lovegrove, 2003). Here, thermal conductance, otherwise heat loss rate, was manipulated via feather insulation by trimming the feathers around the brood patch and those covering pectoral muscles. Feather-clipping took place on day 6 post-hatching, while resting metabolic measurements (RMR) on day 12 to compare the slope regression of the O_2 consumption (heat loss rate) between the feather-clipped and unclipped females. RMR measurements took place for the whole night for 11 hours at ambient temperatures of 14 and 25 °C (which are both below TNZ of the species, (Calder, 1964; Briga and Verhulst, 2017)). To avoid any differences between individuals due to variation within the night of the RMR measurements, the order of the temperatures were randomized (from 14 to 25 °C or 25 to 14 °C). After 6 hours the temperature was switched to the other one and took around 5 minutes to reach the desired temperature.

Before RMR, body mass of each female was measured and afterwards females were placed into sealed individual chambers and later inside a dark climatic "room". Individual chambers were built from typical commercial glass containers of 1100 ml volume and painted externally with black colour to maintain dark conditions. Inside, the individual chamber a metal cage was place for restraining birds from flight movements and attached to the cage a wooden stick for

allowing birds to perch during the measurements. An inlet tube was inserted inside the chamber around 2 cm above the bottom and outlet at the top which allowed a good air flow inside the chamber. The bottom of the individual container was filled with 50 ml of white mineral oil (AnVit, Poland) to collect faeces. A fresh sample of air in standard pressure and room temperature was dried with silica gel driers and divided into 8 streams; seven was pumped into chambers with eight mass flow system pumps 2LPM (Sable System International, USA), and one for baseline reference. Air flow was set at 500 ml min-1 through chambers and regulated separately for each chamber. Samples from chambers were synchronized sequentially through Intelligent Multiplexer MUX (Sable System International, USA). Sub-sample of air stream was first analysed for water content and then was pre-dried using permeable-membrane dryer and then dried with magnesium perchlorate (Anhydrone, J.T. Baker, USA) columns before passing the CO_2 and O_2 analyser. Gas concentrations were recorded every one second through a Field Metabolic System and were analysed and downloaded through ExpeData software (Sable System International, USA). Representative O_2 concentration values were calculated for each bird from the values recorded at the last 20 seconds just before switching the channels similar to (Sadowska et al., 2015) for both temperatures. The last five cycles of the measurements were accounted for each ambient temperature to obtain the minimum value of metabolic rate (criteria for minimum metabolic rates) to assure that birds were under a post-absorptive state.

For the analysis, linear mixed effect model was performed with RMR as response variable, while feather-clip manipulation (feather-clipped versus unclipped), temperature (14 and 25 °C), chamber as predictors and body mass post-absorptive as a covariate. Female identification number was included as random effect to account for the repeated measures of the same individual for the two ambient temperatures. RMR depended on the feather-clip manipulation ($F_{1,35.9} = 4.2$, $p = 0.04$); feather-clipped females revealed higher RMR than the unclipped ones and this result was similar for both temperatures (Figure 16). There was a strong

evidence that ambient temperature has a strong effect on RMR ($F_{1,36.9} = 664.8$, $p < 0.001$). Body mass also contributed significantly to RMR variation ($t = 3.07$, $F_{1,35.9} = 13.7$, $p < 0.001$). Values of RMR were converted to obtain the energy equivalents in Watts (=KJ/s) according to the following formula (Lighton, 2008, Measuring Metabolic Rates):

$$RMR\,[W] = RMR[ml\,O_2 min^{-1}] \times (16 + 5.164 \times RQ)/\,60$$

where RQ (respiratory quotient) was calculated as

$$RQ = VCO_2 \,/\, VO_2.$$

At ambient temperature of 25 °C, feather-clipped females (0.34 ± 0.04 W) lost 0.03 W, by 9.5%, compared to the unclipped females (0.31 ± 0.03 W). The unclipped individuals' RMR in 25 °C validates the values of (Briga and Verhulst, 2017), from which we acquired RMR (0.31 ± 0.05W, n = 23) in 25 °C (± 0.42). At ambient temperature of 14 °C, feather-clipped females (0.49 ± 0.04W) lost 0.03 W, by 6.5%, in comparison to the unclipped ones (0.46 ± 0.05W). Similarly, I examined Briga and Verhulst (2017) results, finding that RMR in 14 °C (± 0.99) was on average 0.48 (± 0.07), but making it difficult to make further comparisons with the data of this study.

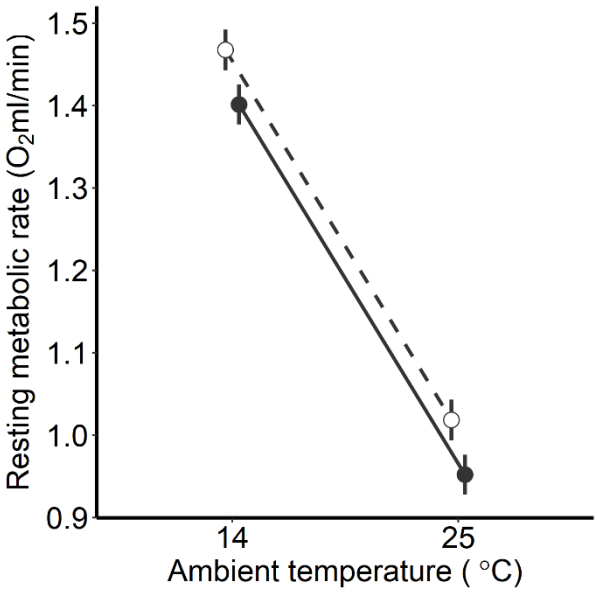

Figure 16. Resting metabolic rate (O2 ml/min) of feather-clipped (open symbols) and unclipped (filled symbols) female zebra finches at 14 and 25 °C. Points are jittered for better visualization and represent least square means and error bars standard error. (see Supplementary Figure 4 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Statistical analysis

All analysis was performed using R computer software (R version 4.1.2, (R, 2021)). For the linear mixed effect models I used *lme4* package (Bates et al., 2015) and *lmerTest* package (Kuznetsova et al., 2017) to calculate the degrees of freedom and p values. Post-hoc comparisons were performed using Tukey method in case of two groups of means, and Sidak method for multiple groups of means. Least square means (LSM) and standard error means (±SE) were calculated using *emmeans* package (Lenth, 2022). I checked for possible interactions between the factors but all non-significant interactions ($p > 0.05$) were removed from the models if not in the interest of the study. All models were checked for normality assumptions and homogeneity of variance by visual inspection of the residuals and in some cases variables, i.e. clutch size, uric acid and oxidative stress index, were log-transformed to

meet the assumptions of normality. All figures presented are based on raw data (if otherwise stated).

Egg mass

I tested differences in egg mass by performing linear mixed effect model analysis, with egg mass the response variable and the ambient temperature as the predictor. Clutch size (the number of eggs) and the body mass of the mother were included as covariates, and the identity of the nest box was included as a random effect to control for any dependence laid on the same nest by the same mother.

Female body mass

I performed one-way ANOVA for testing differences in body mass in mothers at the beginning of the experiment between the ambient temperatures. Linear mixed-effect model analysis was performed for body mass in females including ambient temperature and nestling age (day 0, 4 and 6 - which was the day of feather-clip manipulation) as fixed effects and their interaction. Another linear mixed effect model was performed for analysis of female body mass with the explanatory variables of ambient temperature (factor I; cold and warm), feather-clip manipulation (factor II; feather-clipped, unclipped), the nestling age (day 8, 10, 12, 16, 35) and their interaction. Number of chicks was included as a covariate. For both models, the identity of the female was included as a random effect.

Oxidative status

I tested for differences in oxidative stress biomarkers (response variable) in females between the two ambient temperatures (factor I) performing one-way ANOVA when sampled just before reproduction. Blood samples were also taken from mothers at the peak of food provisioning (day 13) and towards the end of offspring rearing respectively (day 35). The given oxidative stress biomarker was set as a response variable and I added nestling age as a

categorical variable with two levels: day 13 and day 35), ambient temperature and the feather-clip manipulation groups as predictors and their interaction as well. The body mass of the female was included as a covariate, while the identification number of the female was a random effect to account for the repeated measures.

Food intake

I tested for differences in food intake between the two ambient temperature groups before the feather-clip manipulation, at the nestling age of 4 to 6 days old performing ANCOVA. Female body mass and clutch size were included as covariates. After feather-clip manipulation, a linear mixed effect model was performed to detect variation in food intake during the chick development over three 48h periods; 1) from 6 to 8 days old of chicks, 2) from 8 to 10 days old and 3) from 10 to 12 days old. As predictor variables, the ambient temperature, the feather-clip manipulation, the sampling point, and their interaction were added. Brood size and female body mass were included in the model as covariates.

Nestling development

a) To test differences in body mass, tarsus, and wing length (response variables) between the two ambient temperatures (factor I) until the feather-clip manipulation that took place on day 6, I performed linear mixed effect model including interaction between ambient temperature and experiment day. Brood size was also included as a covariate to control for any differences between small and large broods and the identity of the female and the identity of the chicks were set as random effects.

b) I performed linear models over the nestling age since the relationship appeared to be linear (contrary to the asymptomatic growth slope) to test differences in body mass, wing, and tarsus length in nestlings (response variable) between the two ambient temperatures (factor I) and the feather-clip manipulation groups (factor II). For this model I included a third factor of the

experimental day (factor III; levels: day 8, 10, 12, 16 and 35) and the interaction between the three factors (factor I x factor II x factor III). The brood size was included as a covariate to control for any differences in nestling development between small and large broods. The identity of the chicks was included as a random effect to account for the repeated measurements during the development nested in the identity of the mother to control for any dependence in the nestling development from the same mothers.

2.2.3. Results

a) Biparental traits

A total of 82 pairs were exposed to either cold conditions of 14 °C (n = 39) or warm conditions of 25 °C (n = 43). Birds were more successful in laying eggs at the cold compared to warm conditions; about 82% of the 39 pairs (32 out of 39) in the cold and about 51% of the 43 pairs (22 out of 43) in the warm laid eggs ($\chi^2 = 8.67$, $p = 0.003$; Figure 17 A). From those pairs that laid eggs, a total of 26 out 32 chicks hatched in the cold, and 18 out of 22 in the warm. There was no evidence that ambient temperature influenced hatching success ($\chi^2 = 0.002$, $p = 0.95$). When chicks hatched (day 0), there was moderate evidence in clutch size (after log-transformation) between the two ambient conditions ($F_{1,52} = 5.7$, $p = 0.02$; one-way ANOVA, Figure 17 B); females in the warm laid significantly smaller clutches than the females in the cold. Variation in egg mass (ANCOVA) was explained by the ambient temperature ($F_{1,49.5} = 5.7$, $p = 0.02$; Figure 17 C) and female body mass ($t = 2.52$, $F_{1,46.7} = 6.3$, $p = 0.01$), while there was no evidence that the clutch size had an effect on egg mass ($t = 0.91$, $F_{1,37.9} = 0.8$, $p = 0.36$).

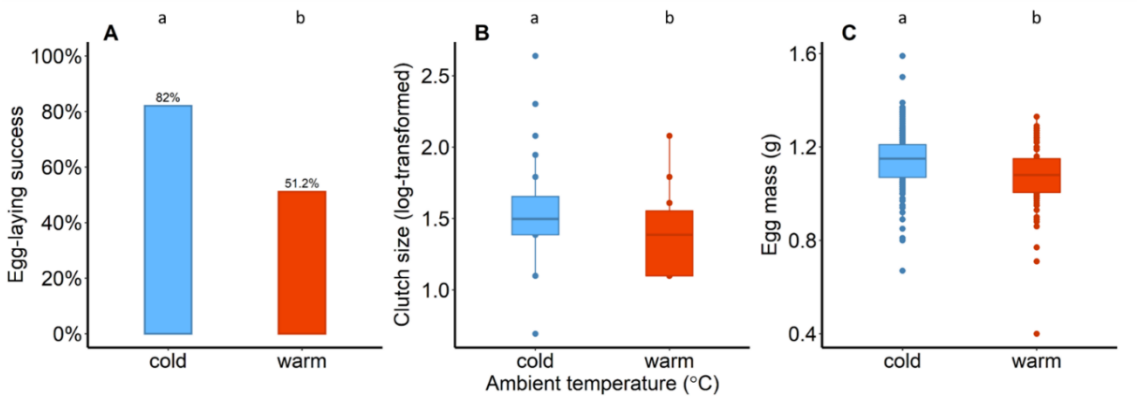

Figure 17. A) Egg-laying success in percentage ($\chi 2$ = 8.67, df = 1, p = 0.003), B) clutch size (after log-transformation) and C) egg mass (g) for the two ambient conditions (cold 14 and warm 25 °C). Different lower-case letters indicate statistical significance. (see Figure 1 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

b) Female traits

Body mass

Initial body mass measured before mating did not differ between the two ambient temperatures ($F_{1,81}$ = 2.28, p = 0.13; one-way ANOVA). Body mass decreased continuously from the day that chicks hatched until day 6 ($F_{2,78.6}$ = 297.8, p < 0.001), but no difference was found between the two ambient temperatures ($F_{1,45.5}$ = 2.72, p = 0.11). Interaction between ambient temperature and nestling age was found to be statistically unclear. Feather-clip manipulation was applied when chicks were 6 days old and there was no evidence that the body mass of the females differ between the experimental groups ($F_{1,38}$ = 2.02, p = 0.16). Linear mixed effect model analysis revealed that there was strong evidence that nestling age was associated with female body mass ($F_{4,157,0}$ = 44.4, p < 0.001); body mass decreased significantly for all females during nestling–rearing period. The data revealed a weak evidence for an interactive effect between feather-clip manipulation and ambient temperature on female body mass ($F_{1,36,9}$ = 3.7,

p = 0.06). Unclipped females in the warm temperature revealed the lowest body mass (11.9 ± 0.4; LSM ± SE) compared to the feather-clipped females in the warm (13.2 ± 0.4; LSM ± SE), unclipped (13 ± 0.3; LSM ± SE) and feather-clipped (12.9 ± 0.3; LSM ± SE) in the cold (Table 5,

Table 6). I also performed ANCOVA for female body mass (g) on day 16 and found moderate evidence for an interactive effect between ambient temperature and feather-clip manipulation ($F_{1,37}$ = 4.26, p = 0.04). Unclipped mothers in the warm temperature revealed the lowest body mass in comparison to the other three groups (Figure 18).

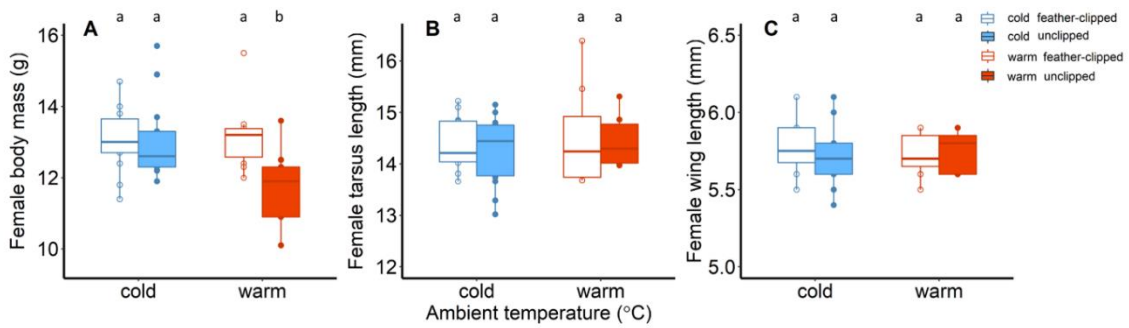

Figure 18. A) Body mass (g), B) tarsus length (mm) and C) wing length (mm) of the females. Body mass of the females is shown at nestling age of 16 days old. Blue color stands for the cold conditions while red for the warm conditions. Filled symbols and boxplots represent unclipped mothers (control) while the open symbols and boxplots the feather-clipped mothers. Boxplots show the median, the range and the inter-quartiles, and the symbols indicate individual values. Different lower-case letters indicate statistical significance. (same figure as Figure 2 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Table 5. Linear mixed model results for the analysis of female body mass (g) and the explanatory variables include ambient temperature (factor I; cold and warm), feather-clip manipulation (factor II; feather-clipped, unclipped), nestling age (day 8, 10, 12, 16, 35), clutch size (day 35), and the interaction between factor I and II. Identification number of the female was included as a random effect. Body mass (g) was recorded just after lights on. Statistics; Estimates, SE: Standard Error, DF: degrees of freedom denominator and p value based on Satterthwaite's method approximation. (same table as Supplementary Table 2 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Variable	Estimate	SE	t	df	p value
Intercept	13.09	0.58	22.67	37.45	<0.0001
Temperature (warm)	0.21	0.50	0.43	36.99	0.67
Manipulation: unclipped	0.12	0.43	0.29	36.99	0.78
day 10	-0.13	0.07	-1.83	159.02	0.07
day 12	-0.28	0.07	-4.10	159.02	<0.0001
day 16	-0.37	0.07	-5.44	159.00	<0.0001
day 35	0.46	0.07	6.70	159.03	<0.0001
number of chicks on day 35	0.07	0.16	0.46	36.99	0.64
Temperature (warm) x Manipulation (unclipped)	-1.43	0.72	-1.97	36.99	0.06
ID female (random)	1.08				

Table 6. Least square means and confidence intervals (CI; lower and upper) adjusted to linear mixed effect model analysis of the female body mass (g) Statistics after post-hoc analysis (Tukey). (same Table as Supplementary Table 3 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

	Feather-clipped		Unclipped		*Estimates*	*se*	*DF*	*t*	*p-value*
	LSM	CI	LSM	CI					
Cold (14 °C)	12.88	(12.29 - 12.88)	13.02	(12.42 - 13.62)	-0.34	0.48	37.00	-0.68	0.49
Warm (25 °C)	13.21	(12.44 - 13.99)	11.90	(11.09 - 12.71)	1.11	0.49	37.00	2.24	0.03

Oxidative – antioxidant status

Effect of temperature before reproduction

There was no evidence for an influence of ambient temperature on uric acid when birds were sampled in the morning, just before mating ($F_{1,72} = 0.61$, $p = 0.43$; Figure 19 A). Similarly, no evidence of ambient temperature in non-enzymatic antioxidant capacity ($F_{1,69} = 0.25$, $p = 0.61$; Figure 19 B). A moderate evidence in oxidative damage, measured as d-ROM, was detected between the two groups of ambient temperature ($F_{1,73} = 5.36$, $p = 0.02$); females in the warm conditions revealed higher oxidative damage (LSM ± SE: 1.75 ± 0.07) than females in the cold conditions (LSM ± SE: 1.49 ± 0.07) (Figure 19 C). Differences between the two ambient temperature in oxidative stress index (after log-transformation), calculated as the ratio of the oxidative damage to the total non-enzymatic antioxidant capacity multiplied by 1000 were not statistically clear ($F_{1,68} = 2.46$, $p = 0.12$; Figure 19 D).

Effect of temperature and feather-clipping during offspring rearing period

There was no evidence for an interactive effect between sample point, feather-clipping and ambient temperature in any of the oxidative stress biomarkers and thus removed these from the models. There was a statistical clear difference in uric acid (after log-transformation) between the two sampling points ($F_{1,38.6} = 65.5$, $p < 0.001$); uric acid in females was higher at the end of the offspring rearing period (LSM ± SE: -0.05 ± 0.07, on day 35) compared to the peak of offspring rearing period (LSM ± SE: -0.76 ± 0.07, on day 13). While ambient temperature did not explain any variation in uric acid ($F_{1,38.2} = 2.9$, $p = 0.09$), there was moderate evidence that feather-clip manipulation had an effect in uric acid ($F_{1,39.0} = 4.6$, $p = 0.03$). Feather-clipped females regardless of the ambient temperature revealed higher uric acid (LSM ± SE: -0.28 ± 0.08) than the un-manipulated (LSM ± SE: -0.53 ± 0.08) females (Figure 19 A). Non-

enzymatic antioxidant capacity also differed significantly between the two sampling points ($F_{1,36.5} = 20.23$, $p < 0.001$); non-enzymatic antioxidant capacity was significantly lower (LSM ± SE: 74.7 ± 5.5) in females towards the end of reproduction in comparison to the peak of offspring rearing period (LSM ± SE: 106.6 ± 5.4; Figure 19 B). However, neither ambient temperature ($p > 0.05$) nor feather-clip manipulation ($p > 0.05$) explained variation in antioxidant capacity. The effect of ambient temperature on oxidative damage was statistically unclear ($F_{1,36.5} = 0.17$, $p = 0.67$) and similarly the effect of feather-clip manipulation ($F_{1,35.9} = 1.1$, $p = 0.29$). There was weak evidence that oxidative damage differed between the two sampling points ($F_{1,40.1} = 3.1$, $p = 0.08$); oxidative damage was slightly higher at the end of offspring provisioning period (LSM ± SE: 1.25 ± 0.05) on day 35 than during the peak of offspring provisioning period (LSM ± SE; 1.13 ± 0.05) on day 13 (Figure 19 C). Oxidative stress index (after log transformation) did not differ between ambient temperature groups ($F_{1,37.39} = 0.87$, $p = 0.35$) and feather-clip manipulation groups ($F_{1,36.58} = 1.34$, $p = 0.25$) but differed between the sampling points ($F_{1,36.9} = 21.91$, $p < 0.001$); oxidative stress index was higher towards the end of offspring-rearing period (LSM ± SE: -4.0 ± 0.09) compared to the peak of offspring-rearing period (LSM ± SE: -4.55 ± 0.09; Figure 19 D).

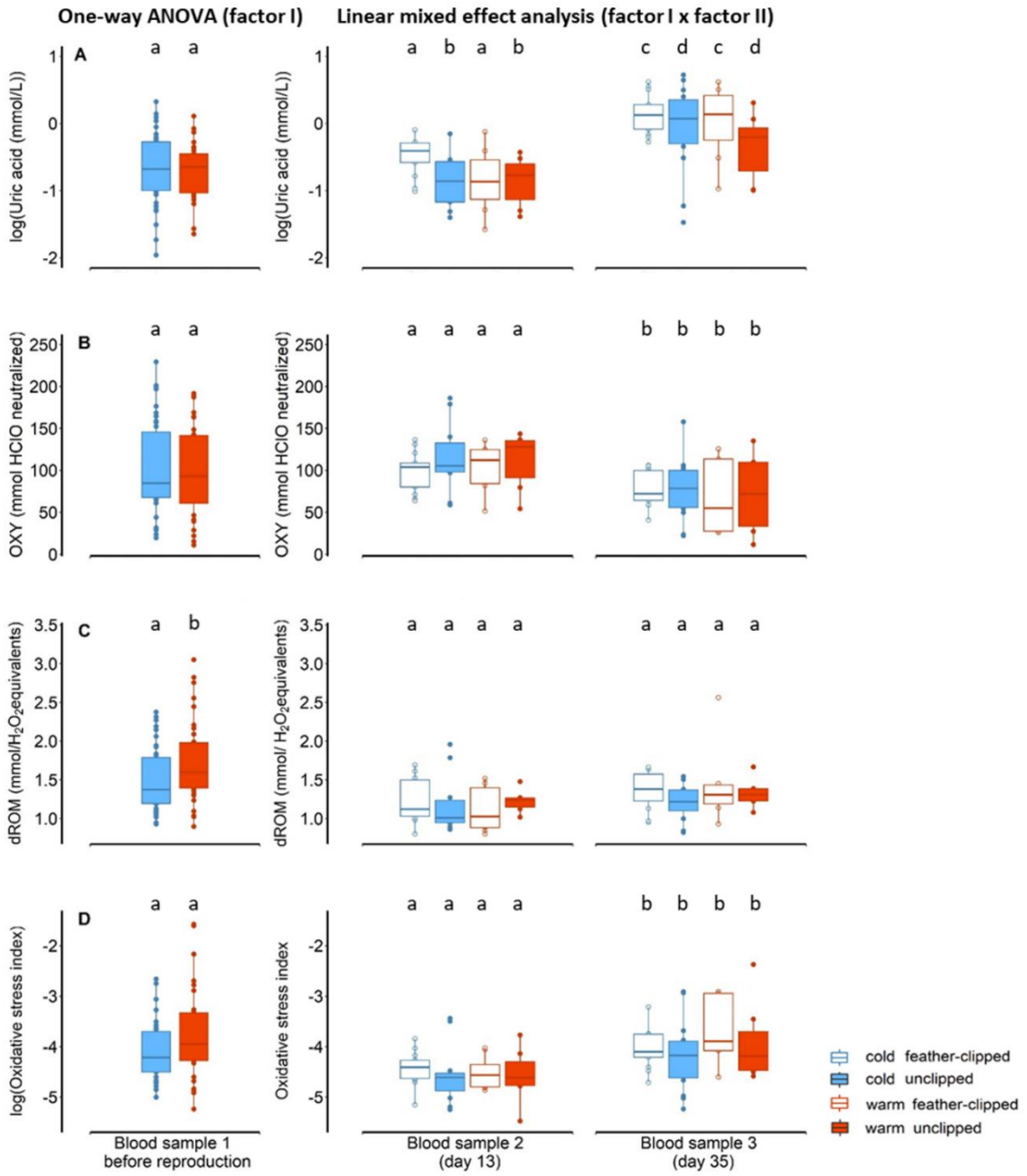

Figure 19. **A)** Uric acid, log-transformed (mmol/L), **B)** OXY (mmol HClO neutralized), a metric for non-enzymatic antioxidant capacity, **C)** dROM (mmol/H_2O_2 equivalents), a metric of oxidative damage and **D)** oxidative stress index, calculated as the ratio of the oxidative damage to the total non-enzymatic antioxidant capacity multiplied by 1000, in plasma concentration of the mother zebra finches (*Taeniopygia guttata*). At the left panel biomarkers are shown when sampled before reproduction and tested for differences using one-way ANOVA (factor I: ambient temperature) and at the right panel during offspring rearing period at nestling age of 13 and 35 days old (blood sample 2 and 3 respectively) tested with linear mixed effect model (factor I, factor II and blood sampling as a third factor). Blue color stands for the cold conditions while red for the warm conditions. Filled symbols and boxplots represent unclipped mothers (control) while the open symbols and boxplots the feather-clipped mothers. Boxplots show the median, the range and the inter-quartiles, and the symbols indicate individual values. Different lower-case letters indicate statistical significance. (same figure as

Figure 3 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

c) Maternal trait

The data revealed strong evidence that the ambient temperature affects food intake over a 48-hour period ($F_{1,32} = 15.41$, $p < 0.001$; Figure 20); food intake in mothers raising hatchlings from day 4 to 6 at the warm temperature was lower (9.4 ± 1.4 g; LSM ± SE) than food intake in mothers raising hatchlings at the cold temperature (16.1 ± 0.9; LSM ± SE). After the implementation of the feather-clip manipulation, there were statistically clear differences in food intake between the two ambient temperatures ($F_{1,31.5} = 25.59$, $p < 0.001$) and the three sample points ($F_{1,84.5} = 46.59$, $p < 0.001$). However, there was no evidence that feather-clip manipulation had an effect in food intake from the mothers ($F_{1,31.5} = 0.01$, $p = 0.91$). There was no evidence for a three-way interaction between factor I, factor II and sample point ($p = 0.44$). Brood size explained most of the variation in food intake ($F_{1,34} = 20.5$, $p < 0.001$). Food intake of the females increased along the development of the chicks, and it was lower for the females breeding in the warm conditions compared to the higher food intake of the females breeding in the cold conditions (Figure 20).

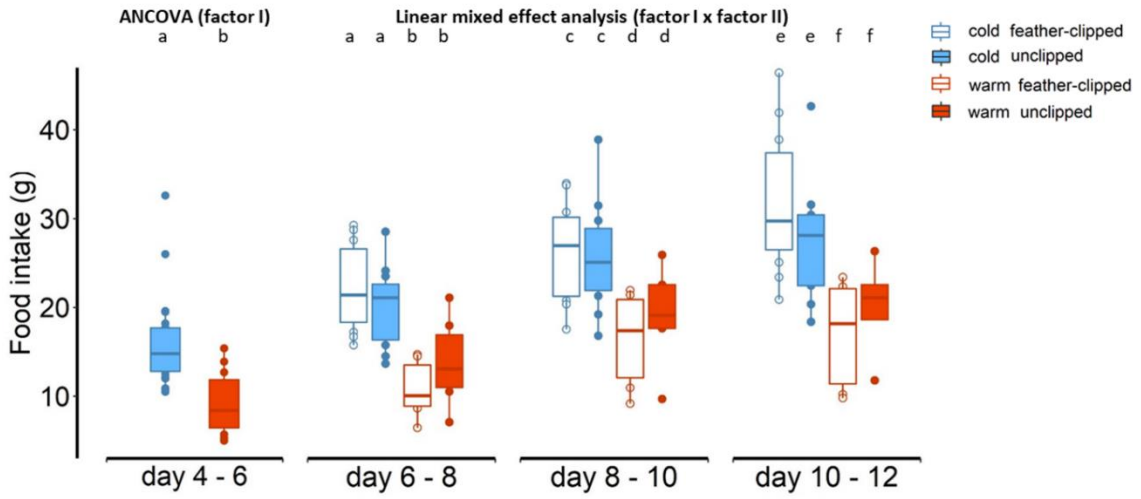

Figure 20. Food intake within 48-hour period of the mothers between cold and warm conditions and after the feather-clip manipulation at day 6 food intake between feather-clipped and unclipped mothers raising chicks at the warm (red color) and cold (blue color) ambient temperature. Different lower-case letters indicate statistical significance. (same figure as Figure 4 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

d) Nestlings

There was no evidence that body mass of the hatchlings differ between the ambient temperature conditions ($F_{1,47.2} = 0.27$, $p = 0.6$) from the day that eggs hatched (day 0) until the day of the feather-clip manipulation (day 6). The interaction between ambient temperature and nestling age was statistically unclear ($F_{1,271.3} = 0.74$, $p = 0.47$). There was strong evidence that body mass of the nestlings differed with the nestling age ($F_{1,271.3} = 491.54$, $p < 0.001$) and there was moderate evidence that the brood size had an effect on body mass ($F_{1,55.37} = 4.29$, $p = 0.04$). After the feather clip manipulation (day 6), the data showed a moderate evidence for an interaction between ambient temperature (factor I), feather-clip manipulation (factor II) and the nestling age (factor III); factor I x factor II x factor III, ($F_{4,465} = 3.64$, $p = 0.01$, see Figure 21, Figure 22) on nestling body mass. At the age of 35 days old, nestlings raised from unclipped females at warm ambient temperature had lower body mass compared to feather clipped

females at the warm temperature compare to all three other groups, while no differences were detected during the nestling development (Figure 21, Figure 22, Table 7, Table 8). Chick mass depended also on the brood size ($F_{1,39.8}$ = 5.8, p = 0.02; Table 7, Table 8).

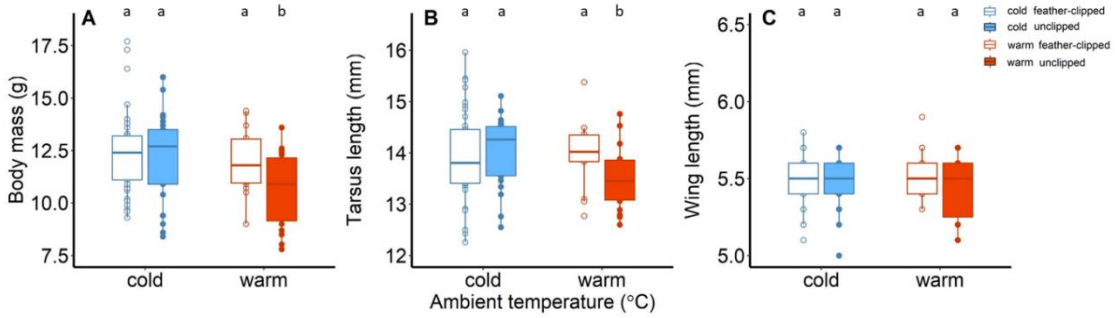

Figure 21. **A)** Body mass (g), **B)** tarsus length (mm) and **C)** wing length (mm) of the nestlings raised in cold (14 °C, blue color) and warm conditions (25 °C, red color) on day 35. Filled symbols and boxplots represent nestlings raised by the unclipped mothers (control) while the open symbols and boxplots nestlings raised by the feather-clipped mothers. Boxplots show the median, the range and the inter-quartiles, and the symbols indicate individual values. Different lower-case letters indicate statistical significance. (same figure as Figure 5 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

For the tarsus length of the chicks, there was a statistically clear interaction between ambient temperature and feather-clip manipulation (factor I x factor II, $F_{1,115.5}$ = 9.07, p = 0.003). Chicks raised from the unclipped females at warm conditions had smaller tarsus length (mm) in comparison to the other three groups; warm feather-clipped, cold unclipped and cold feather-clipped (Figure 21). There was a strong evidence that tarsus length was positively related to nestling age (tarsus length increased with increasing age from 16 to 35 days old; $F_{1,102.97}$ = 88.96, p < 0.001), while brood size on day 35 did not explain any variation of tarsus length ($F_{1,117.06}$ = 9.07, p = 0.76). On the other hand, wing length was not affected by either of

the two manipulations (factor I and factor II) but significantly related to nestling age; wing length was higher with increasing age (from 16 to 35 days old; $F_{1,114.59} = 2233.67$, $p < 0.001$).

Table 7. Adjusted least squares means (LSM) of nestling body mass (g) for each measured day and confidence intervals (CI; lower and upper), corrected for repeated measurements and with covariates fixed at their respective mean. Feather-clip manipulation of mothers took place at nestling age of 6 days old. (same table as Supplementary Table 4 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Day	Cold (14 °C)				Warm (25 °C)			
	feather-clipped		unclipped		feather-clipped		unclipped	
	LSM	CI	LSM	CI	LSM	CI	LSM	CI
0	-	-	0.9	(0.8 - 1.1)	-	-	0.8	(0.6 - 1.0)
4	-	-	2.2	(2.0 - 2.3)	-	-	2.1	(1.8 - 2.3)
6	-	-	2.9	(2.7 - 3.0)	-	-	2.9	(2.7 - 3.1)
8	3.9	(3.3 - 4.4)	3.9	(3.3 - 4.5)	3.9	(3.1 - 4.7)	4.2	(3.4 - 5.0)
10	5.1	(4.6 - 5.7)	5.2	(4.6 - 5.8)	4.9	(4.1 - 5.7)	5.6	(4.8 - 6.4)
12	6.3	(5.7 - 6.8)	6.6	(6.0 - 7.2)	6.2	(5.4 - 7.0)	7.0	(6.1 - 7.8)
16	8.5	(7.9 - 9.0)	8.9	(8.3 - 9.5)	8.4	(7.6 - 9.2)	8.1	(7.3 - 9.0)
35	12.3	(11.7 - 12.9)	11.9	(11.2 - 12.5)	11.8	(10.9 - 12.6)	10.8	(10.0 - 11.6)

Table 8. Linear mixed model results for the analysis of offspring body mass (g) and the explanatory variables include ambient temperature (factor I; cold and warm), feather-clip manipulation (factor II; feather-clipped, unclipped), nestling age (factor III; day 8, 10, 12, 16, 35), clutch size (day 35), body mass of the mothers (g) and the interaction between factor I x II x III. Statistics; Estimates, SE: Standard Error, DF: degrees of freedom denominator and p value based on Satterthwaite's method approximation. (same table as Supplementary Table 5 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

Variable	Estimate	SE	t	df	p value
Intercept	3.71	1.7	2.19	133.51	0.03
Temperature (warm)	-1.04	0.52	-2	62.33	0.05
Manipulation: Unclipped	-0.93	0.6	-1.55	70.68	0.13
day 10	1.43	0.27	5.24	457.56	0
day 12	2.8	0.29	9.67	463.1	0
day 16	3.94	0.28	14.2	467.83	0
day 35	6.65	0.28	23.36	465.49	0
Female body mass	0.27	0.1	2.67	137.13	0.01
number of chicks on day 35	-0.35	0.14	-2.49	39.83	0.02
Temperature (warm) x clipped x Day 8	-2.28	0.84	-2.72	105.22	0.01
Temperature (cold) x unclipped x Day 8	-1.21	0.42	-2.87	458.14	0
Temperature (cold) x unclipped x Day 8	-1.3	0.36	-3.61	457.77	0
Temperature (cold) x clipped x Day 10	-2.44	0.84	-2.91	104.8	0
Temperature (warm) x clipped x Day 10	-1.63	0.42	-3.87	455.52	0
Temperature (cold) x unclipped x Day 10	-1.47	0.36	-4.07	461.23	0
Temperature (cold) x clipped x Day 12	-2.68	0.84	-3.18	107	0
Temperature (warm) x clipped x Day 12	-1.73	0.43	-4.01	456.4	0
Temperature (cold) x unclipped x Day 12	-1.46	0.37	-3.94	460.41	0
Temperature (cold) x clipped x Day 16	-1.6	0.84	-1.9	105.14	0.06
Temperature (warm) x clipped x Day 16	-0.66	0.42	-1.57	453.56	0.12
Temperature (cold) x unclipped x Day 16	-0.3	0.36	-0.85	454.46	0.4
Temperature (cold) x clipped x Day 35	-0.5	0.74	-0.68	64.59	0.5

e) Body mass of the females and nestlings along the nesting-provisioning period

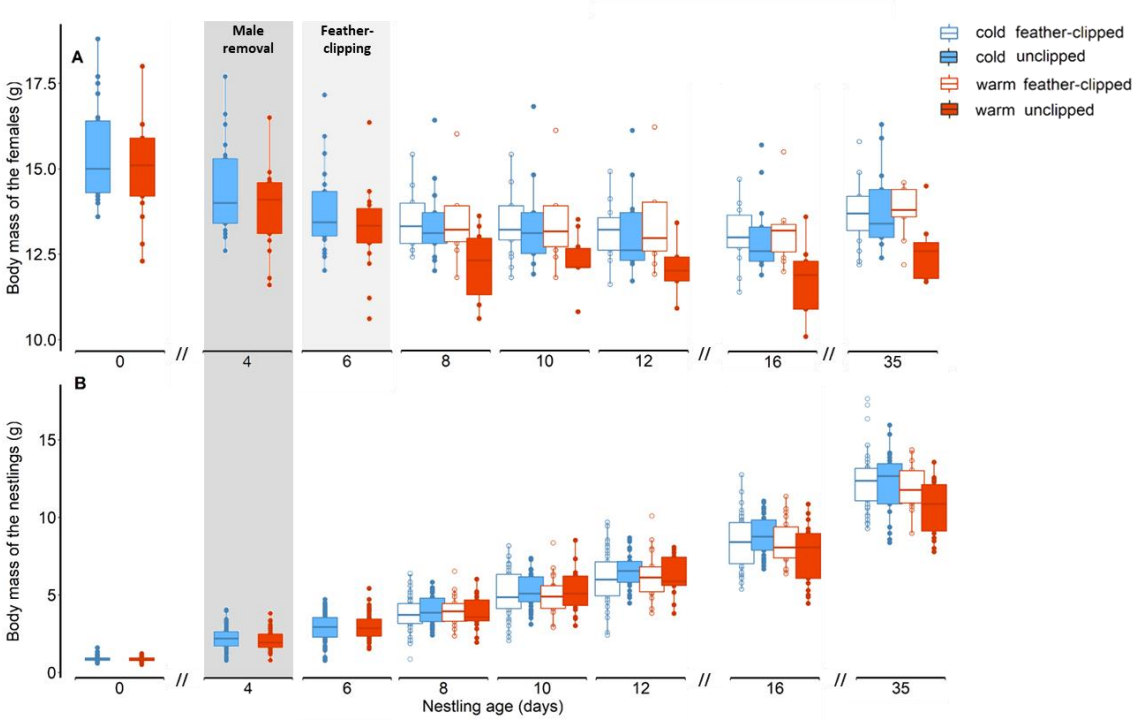

Figure 22. A) Female body mass (g) from the hatching date (day 0) until offspring development (day 35) between cold (blue boxplots) and warm (red boxplots) ambient temperatures, and between feather-clipped (open boxplots) and unclipped (filled boxplots). B) Nestling development under cold (14 °C) and warm (25 °C) conditions and those raised from females in which they were feather-clipped (open points/boxplots) and unclipped (filled points/boxplots). Total 153 nestlings on day 0 from total 46 nests (cold; n = 100 and warm; n = 53), total 142 nestlings from 43 nets on day 6 which feather-clip manipulation took place (cold and feather-clipped; n = 50, cold and unclipped; n = 45 and warm and feather-clipped; n = 22, warm and unclipped; n = 25). Total 114 nestlings from 42 nests on day 35 (cold and feather-clipped; n = 44, cold and unclipped; n = 33 and warm and feather-clipped; n = 18, warm and unclipped; n = 19). (same Figure as Supplementary Figure 5 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).

CHAPTER III General Discussion

"*After all evolution isn't about advancement; it's about survival. It's about learning to solve problems of your environment, something birds have done surpassingly well for a long …long time.*"

J. Ackerman, 2017 (The Genius of Birds)

a) Energetics and limitations during reproduction

From **Experiment I**, I observed female great tits (*Parus major*) raising enlarged brood size with higher daily energy expenditure (DEE) by 12.8% compared to females raising control broods. However, night-time basal metabolic rate (BMR) remained unchanged (Figure 6). Brood size manipulation typically increases DEE, an outcome previously shown (Sanz and Tinbergen, 1999; Wiersma and Tinbergen, 2003; Schifferli et al., 2014). Higher DEE and unchanged BMR suggests that females raising enlarged brood size worked harder to meet the enhanced offspring demands than the control ones, investing energy expenditure towards the reproductive demands for offspring provisioning rather than self-maintenance (Vézina et al., 2006; Welcker et al., 2015). Mothers raising enlarged broods tried to compensate the high demands and increased DEE but failed to fully compensate as observed by the offspring development. Nestlings from enlarged broods were smaller in size and lighter in mass in comparison to the control broods (Figure 9, Figure 11), suggesting parental energetic limitations.

High DEE in great tit females was not supported with high BMR, contrary to the hypothesis that increased energy expenditure requires a large metabolic machinery ("increased intake hypothesis"). Nilsson (2002) applied brood size manipulation in marsh tits (*Poecile palustris*) and found a positive relationship between DEE and BMR when chicks were around 14 days old, according to "increased intake hypothesis". A handicap experiment inducing workload for breeding house wrens (*Troglodytes aedon*) also revealed a positive relationship between DEE and BMR (Tieleman et al., 2008). On the other hand, kittiwakes (*Rissa tridactyla*) exhibited lower free triiodothyronine (fT3) which is a proxy for resting metabolic rate, and increased DEE in response to brood enlargement (Welcker et al., 2015) compared to control broods, supporting "compensation" hypothesis as a saving-energy strategy. Pied flycatchers (*Ficedula*

hypoleuca) raising enlarged broods decreased BMR compared to those ones raising control broods but no changes in DEE (Visser et al., 2019), suggesting that female parents were not able to increase energy for daily activities and applied a saving energy strategy by lowering demands for self-maintenance. Brood enlargement increased female DEE in great tits while no changes were detected in BMR (Wiersma and Tinbergen, 2003), a similar outcome to this study (Figure 6) neither supporting "increased intake" nor "compensation" hypothesis. All the aforementioned findings, together with this study, lead to the idea that the different directions in the correlation between DEE and BMR perhaps are caused by species- specific physiological limitations and/or ecological constraints (Careau et al., 2013; Welcker et al., 2015), a rather context-dependent relation.

Extrinsic factors such as food availability and temperature (Nilsson, 2002; Speakman et al., 2003; Schifferli et al., 2014; Welcker et al., 2015), or intrinsic, e.g. digestive capacity for energy assimilation and utilization (Hammond and Diamond, 1997; Speakman and Garratt, 2014; Welcker et al., 2015), may influence DEE or/and BMR independently. Nilsson (2002) suggested that the "increased intake" hypothesis emerges when foraging costs are low for increasing food intake. On the other hand, energy acquisition can be limited by the digestive or organ capacity, and any physiological changes on the organ capacity may influence BMR (Weiner, 1992). For instance, kittiwakes even though increased DEE due to enlarged brood, fT3 – a proxy of BMR – decreased compared to the control brood while food was supplemented suggesting perhaps limitations due to reduced metabolic intensity in liver and kidney (Welcker et al., 2015). Visser et al., (2019) reported that in flycatchers BMR was not affected when brood size manipulation was applied for a long-time period, while BMR decreased with no changes in DEE measured shortly after brood size manipulation. This outcome rather implies physiological limitations for increasing energy expenditure, perhaps such as adjusting organ size (Visser et al., 2019). Apparently, several aspects such as food availability or energy intake

limitations may drive the relationship of DEE and BMR accordingly to the needs of the organisms. Here, the reasons are not immediately clear for the absent association between DEE and BMR. Perhaps, BMR remained unchanged (Figure 6) allowing great tit females to invest towards reproductive demands, keeping the energetic costs for self-maintenance at regular low levels - without an increase - and thus avoiding any further costs (i.e. oxidative damage Figure 7 C). Data of this study may support the idea that any modification of metabolic rates during sustained workload may be adjusted accordingly for mediating the potential costs of reproduction.

For the second experiment (**Experiment II**), I tested whether breeding birds are limited by the capacity to dissipate the excess body heat produced during sustained energy expenditure. Reduced egg mass and clutch size at the warm conditions (25°C) compared to the cold (14°C) imply that both parents already at the beginning of reproduction are limited by the heat dissipation capacity, in line with the heat dissipation limit hypothesis (HDL, Speakman and Król, 2010b). Breeding pairs at warm temperature were less successful in producing a clutch compared to the breeding pairs at the cold (Figure 17). Reduced egg production in terms of egg number and mass at warm ambient temperature (Figure 17), is consistent with previous findings revealing decreased egg size and mass (Williams and Cooch, 1996; Schaper and Visser, 2013) with increasing ambient temperatures (although see Griffith et al., 2020). Breeding zebra finches showed decreased egg mass and clutch size when exposed to a high foraging cost feeder similar to the one implemented for the Experiment II (Yap et al., 2021), suggesting that energy used was traded-off between foraging costs and egg production. The reduced clutch size and egg mass in the warm conditions observed in Experiment II rather resulted from a combination of both warm temperature and elevated foraging costs. Physical exercise increases heat production and thus combined with warm ambient temperature during

reproduction resulted in lower reproductive output suggesting that the capacity of heat dissipation is indeed important.

By feather-clipping females around the brood patch and the pectoral muscles, heat dissipation capacity was enhanced up to 0.03 W (9.5%) compared to the unclipped females (See *Thermal conductance,* Figure 16). All mothers gradually decreased their body mass during nestling rearing period (Figure 22), suggesting an energetic investment towards reproduction at the cost of self-maintenance (life-history trade-off). However, un-manipulated mothers in the warm revealed lower body mass when nestlings reached 16 days old compared to the other three groups of mothers eased of heat constrains (Figure 18). This suggests that unclipped mothers in the warm were limited by the capacity to heat dissipate. The differences in body mass cannot be explained from the food intake, since food intake, even though dependent on ambient temperature, was independent of the feather-clip manipulation (Figure 20). Here, food intake estimation might be masked by the fact that direct measurements of the food acquired from the female parents and the food passed on to the nestlings are lacking. Body mass loss during reproduction has been previously observed in birds and it has been formulated as a consequence of either a consumption of the energy reserves ("cost of reproduction" hypothesis) or an adaptation to lower the flight costs of adults during nestling rearing ("mass-adjustment" hypothesis) (Hillström, 1995). In this study, birds had unlimited access to food during a 13-hour period of daylight and thus the hypothesis that birds were not able to replenish their energy stores may not be supported by the "cost of reproduction" hypothesis.

Maternal feather-clip manipulation in blue tits (*Cyanistes caeruleus*) enhanced heat loss by 0.09 W and revealed lower body mass loss in feather-clipped females compared to the unclipped ones when nestlings were at 14 days old (Nord and Nilsson, 2019). This is in line to the results of this present study, even though the difference in heat loss is smaller (0.03 W). Higher energy expenditure induced by an experimentally enlarged brood size was negatively

related to body mass in great tits (*Parus major*) (Nadav, 1984), and Nord and Nilsson (2019) suggested that unclipped females may have lost more body mass due to the higher energetic costs of panting to dissipate heat compared to the feather-clipped ones. Panting is a known mechanism in passerines for heat dissipation and more likely to take place during high ambient temperatures for increasing heat loss (McKechnie and Wolf, 2019; Pessato et al., 2020; Oswald et al., 2021). Also, panting requires a high amount of water expenditure causing changes in blood chemistry (Calder and Schmidt-Nielsen, 1966; Schmidt-Nielsen, 1972), and perhaps unclipped mothers in the warm in our study may have used more energy to dissipate heat via panting compared to the clipped mothers in the warm (and both experimental groups in the cold) explaining the body mass difference. Body temperature rises during exercise (Ward et al., 1999; Guillemette et al., 2016) and this rise becomes more pronounced with increasing ambient temperatures (Tucker and Noakes, 2009; Tapper et al., 2020a). When body temperature reaches lethal levels as shown in tree-swallows during offspring provisioning period (i.e. 15% of all observations exceeded 43 °C; Tapper et al., 2020a), organisms have evolved different mechanisms to avoid any irreversible physiological damage. For instance, pacing strategy is known in human athletes (Tucker and Noakes, 2009), and eider ducks stop their flights when body temperature rises significantly (Guillemette et al., 2016) pacing strategy in human athletes; see review in Tucker and Noakes, 2009). Lowering the pace may explain high body mass loss in unclipped birds at the warm temperature. Unclipped females breeding in warm conditions, hence with heat dissipation limitations, may have lost body mass as a strategy to lower metabolic heat production. For instance in humans, runners with lower body mass revealed an advantage of decreased heat production and thus were able to run faster than heavy runners under the hot ambient temperature of 35 °C (Marino et al., 2000). In summary, the body mass changes of mothers in response to two manipulations provide strong

experimental support that the capacity to dissipate heat may indeed represent a limit during sustained parental workload.

In conclusion, both experiments indicate that sustained energy expenditure during food provisioning period is limited and cannot exceed certain levels. In **Experiment I**, females from the enlarged broods increased sustained energy expenditure 3.1 x BMR compared to the females from the control 2.6 x BMR. Breeding females tried to compensate the higher energetic demands of the enlarged brood size but energy expenditure was not enough since nestlings were observed with lower body mass, smaller wing length and structural size (Figure 11), in line with the idea that sustained metabolic scope is limited and cannot exceed certain levels (Peterson et al., 1990). Data from the **Experiment II**, confirm that the capacity to dissipate heat is a very likely constraining mechanism that may influence energy expenditure during intensive physical workload. The **Experiment II** which took part under controlled laboratory conditions provides now comprehensive support for the HDL hypothesis especially under warm conditions and adds up to the current evidence accumulated for free-living birds that also support HDL hypothesis (Nilsson and Nord, 2018; Nord and Nilsson, 2019; Tapper et al., 2020b, 2020a).

b) Oxidative stress during reproduction

Any energy allocation and investment during reproduction may influence the production of free radicals (Monaghan et al., 2009), increasing the risk for encountering oxidative stress (see review Table 1). Contrary to the predictions, at the **Experiment I** oxidative damage did not differ between the females raising enlarged and control broods (Figure 7). This is not the first time that oxidative damage is not detected during intensive reproductive activities (see Nussey et al., 2009; Wilson et al., 2012; Costantini et al., 2010; Costantini et al, 2014a; Markó et al., 2011). Actually, reduced oxidative damage is shown in breeding individuals compared to the non-breeding ones (see meta-analysis Blount et al., 2016). Especially under conditions rich in food resources (natural conditions: Fletcher et al., 2013 or laboratory: Costantini et al., 2014b) oxidative damage in breeding individuals is not detected. At the **Experiment II,** oxidative damage was also not detected during reproduction in breeding zebra finches. The absence of oxidative damage during reproduction observed in some cases, including the present studies, may be due to the fact that antioxidant repair processes take part in the first line of defense (Vaanholt et al., 2008). Animals have evolved the ability to cope with environmental and metabolic changes that promote ROS formation by upregulating endogenous antioxidants (via activation of an electric transcription factor) (Giraud-Billoud et al., 2019; Ensminger et al., 2021). Antioxidants, such as uric acid, glutathione, vitamin C and E, participate in a chain reaction with ROS transforming them to non-reactive and non-harmful products, hence organisms avoid further oxidative damage. The absence of oxidative damage in both studies during reproductive activities indicate that antioxidant mechanisms took over for diminishing oxidative damage produced at the early phase of oxidative cascade.

Several alternative reasons can be put forward to explain the lack of effect on oxidative damage from increased workload in both experiments (**Experiment I, Experiment II**). One reason, for

example, could be the time of sampling that blood was collected. All blood samples were standardized and collected early in the morning after night-time resting activity. It has been proposed that the accumulation of free radicals along the day-time metabolism and activity is removed and quenched during sleep (Reimund, 1994; Wilking et al., 2013), suggesting that any oxidative damage occurred during day-time due to the intensive activity for offspring-rearing could be mitigated during the resting phase. A second explanation could be that oxidative damage in plasma, as measured here, may not always represent the oxidative damage in tissues (Speakman and Garratt, 2014). As previously shown, the direction of oxidative damage may differ between serum and liver (Xu et al., 2014). In Table 1, we may also observe the contradictory findings on oxidative damage that can be related to the different measurements of markers or/and tissues. On the other hand, we should not exclude the idea that high levels of energy expenditure may not necessarily increase reactive oxygen species (ROS), since ROS are not generated in direct proportion to oxygen consumption but rather the mitochondrial coupling state can be the key regulator (Speakman et al., 2004, 2015; Barja, 2007; Speakman and Garratt, 2014; Stier et al., 2014). For instance, increased sustained metabolic rate reduced ROS production (Salin et al., 2015), while reductions in metabolism via fasting increased oxidative stress in brown trout (Salin et al., 2018), contrary to the expected positive relationship between aerobic metabolism and ROS production. Consequently, it is generally important to keep in mind that any type of damage can be reversed and depends on the capacity to repair or other factors. Such damages and/or the repair rate along reproductive activities still require to be explored.

Regarding the antioxidant capacity, at **Experiment I**, we observe a negative relationship between DEE and the total non-enzymatic antioxidant capacity (Figure 9). This suggests that females increased energy investment towards reproductive output and reduced energetic investment in self-maintenance processes. Elevated metabolism perhaps increased the amount

of free radicals and there was need to counterbalance those radicals via the antioxidant defense mechanism. Female barn swallows (*Hirundo rustica*) were detected with decreased antioxidant capacity when nestlings were 15 days in response to brood enlargement, and no changes in oxidative damage (Costantini et al., 2014a). Antioxidant capacity decline was also observed in breeding Seychelles warblers (*Acrocephalus sechellensis*) during the provisioning period compared to the stage before even the nesting phase (van de Crommenacker et al., 2012). Antioxidants, both enzymatic or non-enzymatic, can minimize harmful effects of ROS production generated through intensive physical activity. Homing pigeons after an endurance flight of 200 km also revealed a decreased amount of non-enzymatic antioxidants, indicating the use of antioxidants for compensating the increased amount of ROS production during intensive physical activity (Costantini et al., 2008). On the other hand, handicapped great tit males with increased flight costs, had higher non-enzymatic antioxidants than the control non-handicapped (Casagrande and Hau, 2018). Antioxidants whether enzymatic or non-enzymatic antioxidants may play a different role given the conditions. Results from **Experiment I** rather suggest that the increased energy expenditure (Figure 9 and Figure 8) resulted in a net loss of antioxidants as they were used for quenching free radicals.

In line with this outcome, non-enzymatic antioxidant capacity of breeding females at the **Experiment II** was lower towards the end of nestling-rearing period compared to the peak regardless ambient temperature and feather-clipping (Figure 19 B), confirming that the antioxidant capacity can be up-regulated for the reproductive activities (Costantini et al., 2014a). During intensive physical activities such as reproduction, organisms use antioxidants for maintaining oxidative balance. *Ad libitum* access to food during laboratory conditions gave the opportunity of the mothers to upregulate their antioxidant defenses (Speakman and Garratt, 2014), thus avoiding the risk of further oxidative stress. An opposite pattern in uric acid was detected with higher uric acid towards the end of reproduction. In birds, uric acid has been

suggested to act as endogenously produced antioxidant when oxidative stress is encountered (Tsahar et al., 2006; Alan and McWilliams, 2013). For example, uric acid was found to be positively related with allantoin (its' oxidative product) in white-crowned sparrows during intensive exercise (Tsahar et al., 2006). High levels of uric acid were observed in godwits prior to migratory flight, which perhaps was caused from the breakdown of proteins that originate from body tissue (Gutiérrez et al., 2019). Perhaps, when antioxidant capacity was reduced, then the uric acid took over towards the end of the reproduction and most of the variation was explained mainly by the feather-clip treatment regardless ambient temperature (Figure 19). Feather-clipped females due to higher energetic investment to raise large and heavier offspring (higher reproductive output) entailed catabolism of proteins possibly due to lower investment for self-maintenance, and uric acid increased to diminish any further physiological costs. Both oxidative stress biomarkers in zebra finches, non-enzymatic antioxidant capacity and uric acid, suggest that mothers during offspring-provisioning period did encounter oxidative stress and thus drove changes for the upregulation of antioxidant capacity and uric acid to protect from further costs.

I also estimated oxidative stress index, as the ratio of d-ROMs and OXY, an informative index taking account both functions of the pro-oxidants and the antioxidants in plasma as previously done (Costantini et al., 2006; Markó et al., 2011). For **Experiment I**, oxidative stress index was higher for females raising enlarged broods compared to the control females (Figure 7 D). Moreover, we observed a positive link between sustained metabolic scope and oxidative stress index (Figure 8); females with increased sustained metabolic scope revealed with higher levels of oxidative stress. A similar pattern was observed at **Experiment II,** where oxidative stress index was higher at the end of reproduction compared to the peak of food provisioning period. These findings are in line with the "oxidative cost" hypothesis, that oxidative stress may

actually act as a physiological cost during reproduction ("oxidative cost" hypothesis), based on the idea that elevated metabolism generates a high amount of ROS (Monaghan et al., 2009).

To sum up, rearing offspring requires hard work and time is limited to compensate the needs for soma-maintenance (Tinbergen and Verhulst, 2000; van de Crommenacker et al., 2012). Breeding females at **Experiment I** with higher sustained metabolic scope were detected with lower antioxidant capacity increasing the risk to encounter oxidative stress (Figure 8). Even though there was no evidence that oxidative stress biomarkers differ between the experimental groups (feather-clipping and ambient temperature) during reproductive activities, non-enzymatic antioxidant capacity decreased and oxidative stress index increased for all females substantially towards the end of reproduction in **Experiment II**. When environmental and metabolic changes increase ROS production, endogenous antioxidants are activated (via a transcription nuclear erythroid 2-related factor 2; Nrf2) to diminish those ROS (Giraud-Billoud et al., 2019; Ensminger et al., 2021). Breeding females in both studies worked hard for reproduction and may have "consumed" the endogenous stored antioxidants (i.e. glutathione, vitamin C etc.) for deactivating ROS into non-reactive and less harmful products, lowering the risk for any further oxidative damage.

c) Thermal limitations and oxidative stress

Since metabolic rate is affected by ambient temperature (Scholander et al., 1950), ROS production is also likely to be affected by the environmental temperature (Selman et al., 2000; Blagojević, 2007; Monaghan et al., 2009). At the beginning of **Experiment II**, when zebra finches were sampled just before reproduction revealed higher levels of oxidative damage at the warm ambient temperature than the cold temperature (Figure 19), and this may resulted from the combination of increased physical activity and the warm ambient temperature. Acute exposure in high ambient temperatures and/or simultaneous physical activity may enhance

oxidative stress (Lin et al., 2006; Mestre-Alfaro et al., 2012). Exercise produces heat and zebra finches in Experiment II increased physical activity due to the high foraging cost feeder (Koetsier and Verhulst, 2011; Yap et al., 2017). The combination of exercise with warm ambient temperature likely caused a surplus of body heat and this may explain the higher levels of oxidative damage compared to the cold before the reproductive event (Figure 19 °C).

However, oxidative damage and non-enzymatic antioxidant capacity during food provisioning period appeared unaffected by both manipulations, such as ambient temperature and feather clipping. Similarly to this study, Mongolian gerbils (*Meriones ungulicatus*) exposed to 10, 21 and 30 °C during reproduction also did not reveal differences in various markers of oxidative stress (Yang et al., 2013). High levels of oxidative damage were detected in zebra finches when exposed in high temperatures (38 and 42 °C) during their adulthood, but this effect did not appear in birds that experienced heat-exposure in their early life (Costantini et al., 2012). Previous experience or duration (acute or chronic) of the exposure to the temperature may explain the fact that we detected differences in oxidative damage at the early stage of the experiment but not later during the reproductive events. Perhaps, the oxidative damage that birds experienced at the beginning, through increased physical activity at warm ambient temperatures, may have driven changes in the line of the antioxidant defense mechanisms, such as the non-enzymatic antioxidant capacity and uric acid (Ji, 1999; Stier et al., 2019).

I predicted that feather clipping compared to unclipped treatment would remove the thermal limitations on reproductive performance at both ambient temperatures, but results confirm this prediction only for the high and not for the low ambient temperature. Clearly, the difference between clipped and unclipped birds for the high ambient temperature is in agreement with HDL hypothesis, but the effect of clipping at the low ambient temperature may rather hint to opposite thermal constrains. Cold ambient temperature in combination with the feather-clip manipulation may have enhanced heat dissipation so much that birds now face an additional

energetic burden for thermoregulation, namely to remain normothermic and not to cool out to much (Sadowska et al., 2019). Possibly, the activity levels modulate the impact of the feather-clipping treatment and females in the cold must allocate energy to remain warm when they are not active. In high ambient temperature clipping would improve the thermoregulation of the active birds, reducing their costs of thermoregulation. The effects of clipping on the energy balance of active birds in cold temperature and of inactive birds in high temperature should be intermediate. While we observed food intake difference only between warm and cold treatment and not between the feather manipulations, we did observe body mass effects on females and offspring and structural size effects on offspring only in the difference between clipped and unclipped females at the warm ambient temperature and not in the cold. Non-enzymatic antioxidant capacity decreased for all females during nestling-rearing period (Figure 19B), uric acid increased, but this was more pronounced for the feather-clipped females, both in warm and cold temperature, than the control (Figure 19A). These results may suggest the upregulation of the antioxidant defense system could neutralize the adverse effects of oxidative stress (Alan and McWilliams, 2013; Gutiérrez et al., 2019) but the mechanism may differ between the ambient temperatures. The association between reproduction and oxidative stress is rather complex and may also act as a constrain. Experimentally reduction of glutathione, a key antioxidant, in canaries (*Serinus canaria*) delayed the hatching date and affected the clutch size (Costantini et al., 2016), suggesting that indeed oxidative stress may act as a constrain during reproduction. On the other hand, even if females were able to increase energy towards reproductive output avoiding oxidative stress, it may not necessarily be an advantage of the nestlings to gain more mass or develop larger beyond the observed levels (Blanckenhorn, 2000). Thus, it would be interesting to experimentally enhance energetic demands through an enlarged brood size and investigate whether breeding animals released of thermal constraints may raise energy expenditure.

To conclude, this study corroborates HDL hypothesis, but it also indicates that alternative limitations are likely occurring during reproduction and these may even entail a differential view of opposing thermal limitations between active and non-active phases.

d) Trade-offs between soma maintenance and reproductive output

Great tit females at **Experiment I**, even though increased energy expenditure to compensate the nestling demands of the brood enlargement, it did not come with a benefit of higher mass of chicks but rather with lower mass of chicks (Figure 11). Nestlings from the enlarged broods revealed smaller tarsus, wing length, and lower body mass than the nestlings from the control broods (Table 3), a pattern that has been previously observed at brood size manipulations (Moreno et al., 1997; Neuenschwander et al., 2003; Losdat et al., 2010). Parents raising more chicks may not be able to compensate for the high needs of food provisioning, leading to increased competition for food between the siblings (Nilsson and Svensson, 1996; Neuenschwander et al., 2003). Body condition of the nestlings is considered to be positively related to future survival (Linden et al., 1992; Perrins and McCleery, 2001). Heavier in mass fledglings have a greater chance to survive than the fledglings with lean mass (Smith et al., 1988; Perrins and McCleery, 2001), although see (Adriaensen et al., 1998). In addition, competition of food and overall growth rate of the chicks may affect the metabolic costs and perhaps the level of oxidative stress (Costantini and Dell'Omo, 2006b; Losdat et al., 2010). In most cases, costs of reproduction affect offspring development, hence their survival, rather than the survival of the parents (Linden and Møller, 1989; Moreno et al., 1997), suggesting that parents encounter trade-offs between their own survival and reproductive success (current versus future reproduction).

In **Experiment II**, we observed that feather-clipping in female adults not only affected their own body mass over the course of the reproductive event and possibly oxidative stress but also

nestling development and the interactive effect was only detected at the nestling age of 35 days old (Figure 21, Figure 22). At the nestling age of 35 days zebra finches reach almost a fully developed juvenile size and move towards independence from their parents (Zann, 1996). Nestling zebra finches developed in 30 °C ambient temperature were smaller in body mass and tarsus length than those in 18 °C; however, these differences were only visible at the age of 28 days, but not at the age of 12 days (Andrew et al., 2017). This outcome is similar to the finding of Experiment II, in which reduced offspring mass and size were observed at the warm temperature raised by unclipped females on day 35, when nestlings reached nearly fully developed juvenile size, and no differences were observed in the early stages of development. Parental care and offspring development are closely coupled (Ricklefs, 1984; Zann, 1996) and it is unclear if the effect during the last stage of development is due to cumulative effects over the development of the offspring or due to the difficulty to quantify the effects of maternal care at the early stages of nestling development. For instance, the early stage of development is characterized by intensive nestling signals, such as begging calls, that may modify and influence parental care and the level of food provisioning (Leonard and Horn, 2001). Begging calls take part in young zebra finches up to 14-16 days old and after fledgling young ones are less active and beg just before a feeding bout (Zann, 1996). Even though mothers were constrained by the heat dissipation capacity, they were rather modulating maternal care due to nestling signals, hence masking any effects at the early stage. Perhaps, any effects of thermal constrains during sustained parental workload are more likely to shape offspring development at the later stage of development.

Any conditions experienced during a specific biological stage very often impact the performance of the individuals at the subsequent biological stage (Blomberg et al., 2014). When zebra finches bred in two ambient temperatures of 18 and 30 °C, nestlings raised in the hot temperature were lighter and smaller at 28 days than those raised in the cold but the effect

of body mass was removed when they reached 90 days old (complete juvenile size) and only the effect in tarsus remained (Andrew et al., 2017), a partial "catch-up". Here, nestlings raised by the unclipped mothers in the warm differed significantly in body mass and tarsus length compared to the other three groups, while wing length remained unchanged (Figure 5B, 5C). We did not follow their post-juvenile development (as in the study of Andrew et al., 2017) and we do not know whether nestlings were able to catch up with increasing their body mass. Tarsus length represents skeletal size in birds and may act as a "thermal window" which may serve for heat dissipation (Speakman and Ward, 1998). For instance, Allen's rule suggest that small morphological traits should rather reflect an adaptive response to cold climates for heat conservation and large in warm climates for higher heat loss. Reduced body size is associated with warm climates, while increased with cold ones following Bergmann's rule (Ashton, 2002; Cunningham et al., 2013; Kruuk et al., 2015; Andrew et al., 2017; Oswald et al., 2021). However, here we found that nestlings raised by the feather-clipped females in the warm were larger than those raised by unclipped, suggesting that the mechanistic link between the climate experienced during development and the body size is likely driven by the physiological constrains of parental care and food provisioning. Wing length represents both skeletal size and feather growth, which depends on other factors like for example nutritional supply (Senar and Pascual, 1997; Oswald and Arnold, 2012; Andrew et al., 2017). Feathers require necessary nutrients for growth (Hill and Montgomerie, 1994) and nestlings in this study even though we observed differences in body mass and tarsus length, wing length was unaffected that may be explained by the similar amount of nutrients obtained through the seeds. In addition, tarsus size of the nestlings at this stage (35 days old) was more developed close to adult size (Figure 21, Figure 18) while wings were not fully developed (see differences in female and offspring wing length; Figure 21 C, Figure 18 C). Perhaps any effects on wing length could be detected later when wings are fully developed close to adult size. Low mass and small structural size in

nestlings implies lower survival probability (Magrath, 1991; Schwagmeyer and Mock, 2008; Ronget et al., 2018) and Tapper et al., (2020b) show evidence that nestlings with lower mass and size raised from unclipped mothers were less successful to fledge than those raised from the feather-clipped mothers. The observed effects on nestling size and mass provide strong evidence that HDL plays a pivotal role in reproductive success and thus fitness of the organisms.

Offspring development may be affected indirectly by maternal reproductive performance as we observe in both studies (**Experiment I, II**). Nestlings from the enlarged brood size were lighter and smaller than the control ones. While nestlings from un-manipulated mothers in the warm temperature were lighter and smaller compared to the mothers eased of body heat. Warm ambient temperature may constrain sustained energy expenditure during reproduction and experimentally releasing the heat burden through feather clipping does relax the thermal limitations. Heat dissipation capacity appears as limitation of sustained performance. Under the scenario of contemporary climate change, which is characterized by continuously rising ambient temperatures and extreme weather events such as heat waves (Ummenhofer and Meehl, 2017), thermal limitations can be expected to impose even bigger and strenuous challenges for organisms in general, and during reproduction in particular.

MAJOR FINDINGS AND CONCLUSION

This research aimed at unravelling the link between energy metabolism and oxidative stress during reproduction in two model avian species: a free-living and a common captive passerine. Based on the findings in this study, it can be concluded that reproductive activities are antioxidant-demanding. This conclusion is supported by the fact that sustained metabolic scope in great tits (*Parus major*) was negatively associated with antioxidant capacity; females with higher metabolic scope had lower antioxidant capacity. If high metabolic costs come at the cost of lower antioxidant capacity, it may increase the risk of encountering oxidative stress. The fact that females from the enlarged broods lifted metabolic scope indicates that a parent is willing to increase energy expenditure for the offspring requirements, but it may come at a cost of oxidative stress. Also, antioxidant capacity in breeding zebra finches (*Taeniopygia guttata*) decreased significantly towards the end of reproduction compared to the peak of offspring-provisioning period. Since Drent and Daan (1980) have indicated that parents increase energy expenditures four times basal metabolic rate during offspring-rearing period, reaching an energetic ceiling, several hypotheses were tested to identify constraints for sustained energy expenditure. Herein, I suggest that antioxidant depletion during intensive workload may increase the risk of oxidative stress and any further lift of energy expenditure could cause oxidative imbalance. Oxidative stress may potentially act as a constraint to maximize sustained energy expenditure.

I tested heat dissipation limit (HDL) hypothesis under controlled laboratory conditions but simulating natural foraging costs by constructing a high-foraging cost feeder, hence increasing physical activity, despite the typical *ad libitum* access to food. Such a high foraging cost feeder is an important requirement for meaningful testing of HDL hypothesis in captive animals. Findings of this study provide comprehensive support for the HDL hypothesis and adds up to the current evidence accumulated for free-living birds that also corroborate HDL hypothesis

(Nilsson and Nord, 2018; Nord and Nilsson, 2019; Tapper et al., 2020b, 2020a). Bird populations decline at the global scale and it is very likely to get worse, given the current climate change crisis. The continuously increasing ambient temperatures globally and the extreme weather events may pose challenging conditions for the reproductive success of the organisms. This thesis provides further and even stronger evidence that the ability to dissipate heat may indeed constrain individuals to perform at high levels, especially under warn conditions. Thermal limitations can be expected to impose even bigger and strenuous challenges for organisms in general, and during reproduction in particular. What is still unclear, is whether the problem of heat dissipation may jeopardize future maternal or offspring survival. In this regard, one ought to track individual survival to gauge any physiological or behavioural adjustments to thermal limitations.

Both ambient temperature and physical exercise may alter metabolic rate and, subsequently, ROS production, resulting in oxidative damage, if not quenched by the antioxidants. Oxidative damage was higher in female zebra finches under warm conditions compared to the cold. The combination of warm ambient temperature and induced physical activity decreased clutch size, egg mass and egg-laying success but later towards offspring food provisioning, enhancing the ability to dissipate heat the reproductive output increased. Follow-up research is required to understand potential trade-offs between management of oxidative stress under thermal limitations and body condition. Oxidative stress avoidance during reproduction might be the key to understand the allocation of resources to either current or future reproduction. Any challenges due to high temperatures during reproductive events may suppress both current and future reproduction (Andreasson et al., 2020), and thus consequently setting limits to species distribution. Detailed understanding of what limits species distribution and fitness may enable us to tailor conservation approaches that are particularly needed under the current global change scenario.

REFERENCES

Adriaensen, F., Dhondt, A. A., Van Dongen, S., Lens, L., and Matthysen, E. (1998). Stabilizing selection on blue tit fledgling mass in the presence of sparrowhawks. *Proc. R. Soc. B Biol. Sci.* 265, 1011–1016. doi:10.1098/rspb.1998.0392.

Alan, R. R., and McWilliams, S. R. (2013). Oxidative stress, circulating antioxidants, and dietary preferences in songbirds. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* 164, 185–193. doi:10.1016/j.cbpb.2012.12.005.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., and Sorci, G. (2004). Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol. Lett.* 7, 363–368. doi:10.1111/j.1461-0248.2004.00594.x.

Andreasson, F., Nilsson, J. Å., and Nord, A. (2020). Avian Reproduction in a Warming World. *Front. Ecol. Evol.* 8, 1–8. doi:10.3389/fevo.2020.576331.

Andrew, S. C., Hurley, L. L., Mariette, M. M., and Griffith, S. C. (2017). Higher temperatures during development reduce body size in the zebra finch in the laboratory and in the wild. *J. Evol. Biol.* 30, 2156–2164. doi:10.1111/jeb.13181.

Arct, A., Rutkowska, J., Martyka, R., Drobniak, S. M., and Cichon, M. (2010). Kin recognition and adjustment of reproductive effort in zebra finches. *Biol. Lett.* 6, 762–764. doi:10.1098/rsbl.2010.0417.

Ashton, K. G. (2002). Patterns of within-species body size variation of birds: Strong evidence for Bergmann's rule. *Glob. Ecol. Biogeogr.* 11, 505–523. doi:10.1046/j.1466-822X.2002.00313.x.

Barja, G. (2007). Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: Implications for aging studies. *Rejuvenation Res.* 10, 215–223. doi:10.1089/rej.2006.0516.

Bates, D., Mächler, M., Bolker, B. M., and Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67. doi:10.18637/jss.v067.i01.

Beckman, K. B., and Ames, B. N. (1998). The Free Radical Theory of Aging Matures. *Physiol. Rev.* 78, 547–581. doi:10.1089/152308603770310202.

Berman, E., Fortson, S., Snaith, S., M, G., Baer, D., Chery, I., et al. (2012). Direct analysis of δ2H and δ18O in natural and enriched human urine using laser-based, off-axis integrated cavity output spectroscopy. *Anal. Chem.* 20, 9768–9773.

Bertrand, S., Alonso-Alvarez, C., Devevey, G., Faivre, B., Prost, J., and Sorci, G. (2006). Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. *Oecologia* 147, 576–584. doi:10.1007/s00442-005-0317-8.

Blagojević, D. P. (2007). Antioxidant systems in supporting environmental and programmed adaptations to low temperatures. *Cryo-Letters* 28, 137–150.

Blanckenhorn, W. U. (2000). The Evolution of Body Size: What Keeps Organisms Small? *Q. Rev. Biol.* 75, 385–407.

Blomberg, E. J., Sedinger, J. S., Gibson, D., Coates, P. S., and Casazza, M. L. (2014). Carryover effects and climatic conditions influence the postfledging survival of greater sage-grouse. *Ecol. Evol.* 4, 4488–4499. doi:10.1002/ece3.1139.

Blount, J. D., Vitikainen, E. I. K., Stott, I., and Cant, M. A. (2016). Oxidative shielding and the cost of reproduction. *Biol. Rev.* 91, 483–497. doi:10.1111/brv.12179.

Bouwhuis, S., Sheldon, B. C., and Verhulst, S. (2011). Basal metabolic rate and the rate of senescence in the great tit. *Funct. Ecol.* doi:10.1111/j.1365-2435.2011.01850.x.

Briga, M., and Verhulst, S. (2017). Individual variation in metabolic reaction norms over ambient temperature causes low correlation between basal and standard metabolic rate. *J.*

Exp. Biol. doi:10.1242/jeb.160069.

Broggi, J., Hohtola, E., Koivula, K., Orell, M., and Nilsson, J. Å. (2010). Idle slow as you grow old: Longitudinal age-related metabolic decline in a wild passerine. *Evol. Ecol.* 24, 177–184. doi:10.1007/s10682-009-9299-z.

Burness, G. P., McClelland, G. B., Wardrop, S. L., and Hochachka, P. W. (2000). Effect of brood size manipulation on offspring physiology: An experiment with passerine birds. *J. Exp. Biol.* 203, 3513–3520. doi:10.1242/jeb.203.22.3513.

Burton, T., Killen, S. S., Armstrong, J. D., and Metcalfe, N. B. (2011). What causes intraspecific variation in resting metabolic rate and what are its ecological consequences? *Proc. R. Soc. B Biol. Sci.* 278, 3465–3473. doi:10.1098/rspb.2011.1778.

Bury, S., Cichoń, M., Bauchinger, U., and Sadowska, E. T. (2018). High oxidative stress despite low energy metabolism and vice versa: Insights through temperature acclimation in an ectotherm. *J. Therm. Biol.* 78, 36–41. doi:10.1016/j.jtherbio.2018.08.003.

Buttemer, W. A., Bauer, S., Emmenegger, T., Dimitrov, D., Peev, S., and Hahn, S. (2019). Moult-related reduction of aerobic scope in passerine birds. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 189, 463–470. doi:10.1007/s00360-019-01213-z.

Calder, W. A. (1964). Gaseous metabolism and water relations of the zebra finch, Taeniopygia castanotis. *Physiol. Zool.* 37, 400–413.

Calder, W. A., and Schmidt-Nielsen, K. (1966). Evaporative cooling and respiratory alkalosis in the pigeon. *Proc. Natl. Acad. Sci. U. S. A.* 55, 750–756. doi:10.1073/pnas.55.4.750.

Careau, V., Réale, D., Garant, D., Pelletier, F., Speakman, J. R., and Humphries, M. M. (2013). Context-dependent correlation between resting metabolic rate and daily energy expenditure in wild chipmunks. *J. Exp. Biol.* 216, 418–426. doi:10.1242/jeb.076794.

Carey, C. (1996). *Avian Energetics and Nutritional Ecology*.

Casagrande, S., and Hau, M. (2018). Enzymatic antioxidants but not baseline glucocorticoids mediate the reproduction–survival trade-off in a wild bird. *Proc. R. Soc. B Biol. Sci.* 285. doi:10.1098/rspb.2018.2141.

Christe, P., Glaizot, O., Strepparava, N., Devevey, G., and Fumagalli, L. (2012). Twofold cost of reproduction: An increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. *Proc. R. Soc. B Biol. Sci.* 279, 1142–1149. doi:10.1098/rspb.2011.1546.

Cichon, M., and Linden, M. (1990). The timing of breeding and offspring size in Great Tits Pams major. 364–370.

Costantini, D. (2011). On the measurement of circulating antioxidant capacity and the nightmare of uric acid. 321–325. doi:10.1111/j.2041-210X.2010.00080.x.

Costantini, D., Bonisoli-Alquati, A., Rubolini, D., Caprioli, M., Ambrosini, R., Romano, M., et al. (2014a). Nestling rearing is antioxidant demanding in female barn swallows (Hirundo rustica). *Naturwissenschaften* 101, 541–548. doi:10.1007/s00114-014-1190-2.

Costantini, D., Carello, L., and Fanfani, A. (2010). Relationships among oxidative status, breeding conditions and life-history traits in free-living Great Tits Parus major and Common Starlings Sturnus vulgaris. *Ibis (Lond. 1859).* 152, 793–802. doi:10.1111/j.1474-919X.2010.01052.x.

Costantini, D., Casagrande, S., De Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J., et al. (2006). Correlates of oxidative stress in wild kestrel nestlings (Falco tinnunculus). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 176, 329–337. doi:10.1007/s00360-005-0055-6.

Costantini, D., Casasole, G., AbdElgawad, H., Asard, H., and Eens, M. (2016). Experimental

evidence that oxidative stress influences reproductive decisions. *Funct. Ecol.* 30, 1169–1174. doi:10.1111/1365-2435.12608.

Costantini, D., Casasole, G., and Eens, M. (2014b). Does reproduction protect against oxidative stress? *J. Exp. Biol.* 217, 4237–4243. doi:10.1242/jeb.114116.

Costantini, D., and Dell'Omo, G. (2006a). Effects of T-cell-mediated immune response on avian oxidative stress. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 145, 137–142. doi:10.1016/j.cbpa.2006.06.002.

Costantini, D., and Dell'Omo, G. (2006b). Environmental and genetic components of oxidative stress in wild kestrel nestlings (Falco tinnunculus). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 176, 575–579. doi:10.1007/s00360-006-0080-0.

Costantini, D., Meillère, A., Carravieri, A., Lecomte, V., Sorci, G., Faivre, B., et al. (2014c). Oxidative stress in relation to reproduction, contaminants, gender and age in a long-lived seabird. *Oecologia* 175, 1107–1116. doi:10.1007/s00442-014-2975-x.

Costantini, D., Monaghan, P., and Metcalfe, N. B. (2012). Early life experience primes resistance to oxidative stress. *J. Exp. Biol.* 215, 2820–2826. doi:10.1242/jeb.072231.

Cram, D. L., Blount, J. D., and Young, A. J. (2015). The oxidative costs of reproduction are group-size dependent in a wild cooperative breeder. *Proc. R. Soc. B Biol. Sci.* 282. doi:10.1098/rspb.2015.2031.

Cunningham, S. J., Martin, R. O., Hojem, C. L., and Hockey, P. A. R. (2013). Temperatures in Excess of Critical Thresholds Threaten Nestling Growth and Survival in A Rapidly-Warming Arid Savanna: A Study of Common Fiscals. *PLoS One* 8. doi:10.1371/journal.pone.0074613.

Daan, S., Masman, D., and Groenewold, A. (1990a). Avian basal metabolic rates: Their association with body composition and energy expenditure in nature. *Am. J. Physiol. -*

Regul. Integr. Comp. Physiol. 259, 333–340. doi:10.1152/ajpregu.1990.259.2.r333.

Daan, S., Masman, D., and Groenewold, A. (1990b). Avian basal metabolic rates: Their association with body composition and energy expenditure in nature. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 259. doi:10.1152/ajpregu.1990.259.2.r333.

Deerenberg, C., and Overkamp, G. J. F. (1999). Hard work impinges on fitness : an experimental study with zebra finches. 173–179.

Deerenberg, C., Overkamp, G. J. F., Visser, G. H., and Daan, S. (1998). Compensation in resting metabolism for experimentally increased activity. *J. Comp. Physiol. - B Biochem. Syst. Environ. Physiol.* 168, 507–512. doi:10.1007/s003600050171.

Diamond, J., Karason, W. H., Phan, D., and Carpenter, F. L. (1986). Digestive physiology is a determinant of foraging bout frequency in hummingbirds. *Nature* 320, 62–63.

Drent, R., and Daan, S. (1980). The prudent parent. *Ardea* 68, 225–252.

Dushoff, J., Kain, M. P., and Bolker, B. M. (2019). I can see clearly now: Reinterpreting statistical significance. *Methods Ecol. Evol.* 10, 756–759. doi:10.1111/2041-210X.13159.

Ellison, P. T. (2003). Energetics and reproductive effort. *Am. J. Hum. Biol.* 15, 342–351. doi:10.1002/ajhb.10152.

Ensminger, D. C., Salvador-Pascual, A., Arango, B. G., Allen, K. N., and Vázquez-Medina, J. P. (2021). Fasting ameliorates oxidative stress: A review of physiological strategies across life history events in wild vertebrates. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 256. doi:10.1016/j.cbpa.2021.110929.

Fletcher, Q. E., Selman, C., Boutin, S., McAdam, A. G., Woods, S. B., Seo, A. Y., et al. (2013). Oxidative damage increases with reproductive energy expenditure and is reduced by food-supplementation. *Evolution (N. Y).* 67, 1527–1536. doi:10.1111/evo.12014.

Frisard, M., and Ravussin, E. (2006). Energy Metabolism and Oxidative Stress. 29, 27–32.

Fyhn, M., Gabrielsen, G. W., Nordøy, E. S., Moe, B., Langseth, I., and Bech, C. (2001). Individual variation in field metabolic rate of kittiwakes (Rissa tridactyla) during the chick-rearing period. *Physiol. Biochem. Zool.* 74, 343–355. doi:10.1086/320419.

Garland, T., Downs, C. J., and Ives, A. R. (2022). Trade-offs (And constraints) in organismal biology. *Physiol. Biochem. Zool.* 95, 82–112. doi:10.1086/717897.

Giraud-Billoud, M., Rivera-Ingraham, G. A., Moreira, D. C., Burmester, T., Castro-Vazquez, A., Carvajalino-Fernández, J. M., et al. (2019). Twenty years of the 'Preparation for Oxidative Stress' (POS) theory: Ecophysiological advantages and molecular strategies. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 234, 36–49. doi:10.1016/j.cbpa.2019.04.004.

Gosler, A. (1993). *The great tit*. Hamlyn, Lo.

Griffith, S. C., Andrew, S. C., McCowan, L. S. C., Hurley, L. L., Englert Duursma, D., Buchanan, K. L., et al. (2020). Egg size is unrelated to ambient temperature in the opportunistically breeding zebra finch. *J. Avian Biol.* 51, 1–14. doi:10.1111/jav.02356.

Griffith, S. C., Crino, O. L., and Andrew, S. C. (2017). Commentary: A bird in the house: The challenge of being ecologically relevant in captivity. *Front. Ecol. Evol.* 5, 3–6. doi:10.3389/fevo.2017.00021.

Guillemette, M., Woakes, A. J., Larochelle, J., Polymeropoulos, E. T., Granbois, J. M., Butler, P. J., et al. (2016). Does hyperthermia constrain flight duration in a short-distance migrant? *Philos. Trans. R. Soc. B Biol. Sci.* 371. doi:10.1098/rstb.2015.0386.

Gutiérrez, J. S., Sabat, P., Castañeda, L. E., Contreras, C., Navarrete, L., Peña-Villalobos, I., et al. (2019). Oxidative status and metabolic profile in a long-lived bird preparing for extreme endurance migration. *Sci. Rep.* 9, 1–11. doi:10.1038/s41598-019-54057-6.

Hammond, K. A., and Diamond, J. (1997). Maximal sustained energt budgets in humans and animals. *Nature*` 386, 457–462.

Harshman, L. G., and Zera, A. J. (2007). The cost of reproduction: the devil in the details. *Trends Ecol. Evol.* 22, 80–86. doi:10.1016/j.tree.2006.10.008.

Heiss, R. S., and Schoech, S. J. (2012). Oxidative cost of reproduction is sex specific and correlated with reproductive effort in a cooperatively breeding bird, the Florida scrub jay. *Physiol. Biochem. Zool.* 85, 499–503. doi:10.1086/666840.

Hill, G. E., and Montgomerie, R. (1994). Plumage colour signals nutritional condition in the house finch. *Proc. R. Soc. B Biol. Sci.* 258, 47–52. doi:10.1098/rspb.1994.0140.

Hillström, L. (1995). Body Mass Reduction During Reproduction in the Pied Flycatcher Ficedula hypoleuca : Physiological Stress or Adaptation for Lowered Costs of Locomotion? *Funct. Ecol.* 9, 807–817.

Hõrak, P. (2003). When to pay the cost of reproduction? A brood size manipulation experiment in great tits (Parus major). *Behav. Ecol. Sociobiol.* 54, 105–112. doi:10.1007/s00265-003-0608-1.

Ito, F., Ito, T., Suzuki, C., Yahata, T., Ikeda, K., and Hamaoka, K. (2017). The application of a modified d-ROMs test for measurement of oxidative stress and oxidized high-density lipoprotein. *Int. J. Mol. Sci.* 18. doi:10.3390/ijms18020454.

Ji, L. L. (1999). Antioxidants and Oxidative Stress in Exercise. *Proc. Soc. Exp. Biol. Med.* 222, 283–292. doi:10.1046/j.1525-1373.1999.d01-145.x.

Johnson, M. S., and Speakman, J. R. (2001). Limits to sustained energy intake V. EFFECT OF COLD-EXPOSURE DURING LACTATION IN MUS MUSCULUS M. *J. Exp. Biol.* 204, 1967–1977. doi:10.1242/jeb.204.11.1967.

Johnston, S. L., Souter, D. M., Erwin, S. S., Tolkamp, B. J., Yearsley, J. M., Gordon, I. J., et al. (2007). Associations between basal metabolic rate and reproductive performance in C57BL/6J mice. *J. Exp. Biol.* 210, 65–74. doi:10.1242/jeb.02625.

Jones, D. P. (2006). Redefining Oxidative Stress. *Antioxidants Redox Signal.* 8, 1865–1879.

Koetsier, E., and Verhulst, S. (2011). A simple technique to manipulate foraging costs in seed-eating birds. *J. Exp. Biol.* 214, 1225–1229. doi:10.1242/jeb.050336.

Koteja, P. (1996). Limits to the Energy Budget in a Rodent , Peromyscus maniculatus : The Central Limitation Hypothesis. *Phsyiological Zool.* 69, 981–993.

Król, E., Murphy, M., and Speakman, J. R. (2007). Limits to sustained energy intake. X. Effects of fur removal on reproductive performance in laboratory mice. *J. Exp. Biol.* 210, 4233–4243. doi:10.1242/jeb.009779.

Kruuk, L. E. B., Osmond, H. L., and Cockburn, A. (2015). Contrasting effects of climate on juvenile body size in a Southern Hemisphere passerine bird. *Glob. Chang. Biol.* 21, 2929–2941. doi:10.1111/gcb.12926.

Kuznetsova, A., Brockhoff, P. B., and Christensen, R. H. B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models . *J. Stat. Softw.* 82. doi:10.18637/jss.v082.i13.

Lenth, R. (2022). mmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.7.2. https://CRAN.R-project.org/package=emmeans.

Leonard, M. L., and Horn, A. G. (2001). Begging calls and parental feeding decisions in tree swallows (Tachycineta bicolor). *Behav. Ecol. Sociobiol.* 49, 170–175. doi:10.1007/s002650000290.

Lighton, 2008, Measuring Metabolic Rates.

Lin, H., Decuypere, E., and Buyse, J. (2006). Acute heat stress induces oxidative stress in

broiler chickens. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 144, 11–17. doi:10.1016/j.cbpa.2006.01.032.

Linden, M., Gustafsson, L., and Part, T. (1992). Selection on Fledging Mass in the Collared Flycatcher and the Great Tit. 73, 336–343.

Linden, M., and Møller, A. P. (1989). Cost of reproduction and covariation of life history traits in birds. *Trends Ecol. Evol.* 4, 367–371. doi:10.1016/0169-5347(89)90101-8.

Losdat, S., Helfenstein, F., Gaude, B., and Richner, H. (2010). Effect of sibling competition and male carotenoid supply on offspring condition and oxidative stress. *Behav. Ecol.* 21, 1271–1277. doi:10.1093/beheco/arq147.

Lovegrove, B. G. (2003). The influence of climate on the basal metabolic rate of small mammals: A slow-fast metabolic continuum. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 173, 87–112. doi:10.1007/s00360-002-0309-5.

Magrath, R. D. (1991). Nestling Weight and Juvenile Survival in the Blackbird , Turdus merula. *J. Anim. Ecol.* 60, 335–351.

Marino, F. E., Mbambo, Z., Kortekaas, E., Wilson, G., Lambert, M. I., Noakes, T. D., et al. (2000). Advantages of smaller body mass during distance running in warm, humid environments. *Pflugers Arch. Eur. J. Physiol.* 441, 359–367. doi:10.1007/s004240000432.

Markó, G., Costantini, D., Michl, G., and Török, J. (2011). Oxidative damage and plasma antioxidant capacity in relation to body size, age, male sexual traits and female reproductive performance in the collared flycatcher (Ficedula albicollis). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 181, 73–81. doi:10.1007/s00360-010-0502-x.

McKechnie, A. E., and Wolf, B. O. (2019). The physiology of heat tolerance in small

endotherms. *Physiology* 34, 302–313. doi:10.1152/physiol.00011.2019.

Meerlo, P., Bolle, L., Visser, G. H., Masman, D., and Daan, S. (1997). Basal metabolic rate in relation to body composition and daily energy expenditure in the field vole, Microtus agrestis. *Physiol. Zool.* 70, 362–369. doi:10.1086/639616.

Meniri, M., Evans, E., Thompson, F. J., Marshall, H. H., Nichols, H. J., Lewis, G., et al. (2022). Untangling the oxidative cost of reproduction: An analysis in wild banded mongooses. *Ecol. Evol.* 12, 1–17. doi:10.1002/ece3.8644.

Mestre-Alfaro, A., Ferrer, M. D., Banquells, M., Riera, J., Drobnic, F., Sureda, A., et al. (2012). Body temperature modulates the antioxidant and acute immune responses to exercise. *Free Radic. Res.* 46, 799–808. doi:10.3109/10715762.2012.680193.

Metcalfe, N. B., and Monaghan, P. (2013). Does reproduction cause oxidative stress? An open question. *Trends Ecol. Evol.* 28, 347–350. doi:10.1016/j.tree.2013.01.015.

Moe, B., Rønning, B., Verhulst, S., and Bech, C. (2009). Metabolic ageing in individual zebra finches. *Biol. Lett.* 5, 86–9. doi:10.1098/rsbl.2008.0481.

Monaghan, P., Metcalfe, N. B., and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. *Ecol. Lett.* 12, 75–92. doi:10.1111/j.1461-0248.2008.01258.x.

Moreno, J., Potti, J., and Merino, S. (1997). Parental Energy Expenditure and Offspring Size in the Pied Flycatcher Ficedula hypoleuca. *Oikos* 79, 559–567.

Muff, S., Nilsen, E. B., O'Hara, R. B., and Nater, C. R. (2022). Rewriting results sections in the language of evidence. *Trends Ecol. Evol.* 37, 203–210. doi:10.1016/j.tree.2021.10.009.

Nadav, N. (1984). The Consequences of Brood Size for Breeding Blue Tits I . Adult Survival

, Weight Change and the Cost of Reproduction. *J. Anim. Ecol.* 53, 479–496.

Nagy, K. A. (1983). *The Doubly Labeled Water (3HH18O) Method: a guide to its use.* , ed. UCLA Los Angeles: UCLA Publication no 12-1417.

Naya, D. E., Spangenberg, L., Naya, H., and Bozinovic, F. (2013). Thermal conductance and basal metabolic rate are part of a coordinated system for heat transfer regulation. *Proc. R. Soc. B Biol. Sci.* 280. doi:10.1098/rspb.2013.1629.

Neuenschwander, S., Brinkhof, M. W. G., Kölliker, M., and Richner, H. (2003). Brood size, sibling competition, and the cost of begging in great tits (Parus major). *Behav. Ecol.* 14, 457–462. doi:10.1093/beheco/arg025.

Nilsson, J.-åke, and Svensson, E. (1996). The cost of reproduction: A new link between current reproductive effort and future reproductive success. *Proc. R. Soc. B Biol. Sci.* 263, 711–714.

Nilsson, J. Å. (2002). Metabolic consequences of hard work. *Proc. R. Soc. B Biol. Sci.* 269, 1735–1739. doi:10.1098/rspb.2002.2071.

Nilsson, J. Å., and Nord, A. (2018). Testing the heat dissipation limit theory in a breeding passerine. *Proc. R. Soc. B Biol. Sci.* 285. doi:10.1098/rspb.2018.0652.

Nilsson, J. Å., and Råberg, L. (2001). The resting metabolic cost of egg laying and nestling feeding in great tits. *Oecologia* 128, 187–192. doi:10.1007/s004420100653.

Nimse, S. B., and Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Adv.* 5, 27986–28006. doi:10.1039/c4ra13315c.

Nord, A., and Nilsson, J. Å. (2019). Heat dissipation rate constrains reproductive investment in a wild bird. *Funct. Ecol.* 33, 250–259. doi:10.1111/1365-2435.13243.

Olson, C. R., Wirthlin, M., Lovell, P. V., and Mello, C. V. (2014). Proper care, husbandry, and

breeding guidelines for the zebra finch, Taeniopygia guttata. *Cold Spring Harb. Protoc.* 2014, 1243–1248. doi:10.1101/pdb.prot084780.

Oswald, K. N., Smit, B., Lee, A. T. K., Peng, C. L., Brock, C., and Cunningham, S. J. (2021). Higher temperatures are associated with reduced nestling body condition in a range-restricted mountain bird. *J. Avian Biol.* 52, 1–10. doi:10.1111/jav.02756.

Oswald, S. A., and Arnold, J. M. (2012). Direct impacts of climatic warming on heat stress in endothermic species: Seabirds as bioindicators of changing thermoregulatory constraints. *Integr. Zool.* 7, 121–136. doi:10.1111/j.1749-4877.2012.00287.x.

Perrins, C. M., and McCleery, R. H. (2001). The effect of fledging mass on the lives of great tits (Parus major). *Ardea* 89, 135–142.

Pessato, A., McKechnie, A. E., Buchanan, K. L., and Mariette, M. M. (2020). Vocal panting: a novel thermoregulatory mechanism for enhancing heat tolerance in a desert-adapted bird. *Sci. Rep.* 10, 1–11. doi:10.1038/s41598-020-75909-6.

Peterson, C. C., Nagy, K. A., and Diamond, J. (1990). Sustained metabolic scope. *Proc. Natl. Acad. Sci. U. S. A.* 87, 2324–2328. doi:10.1073/pnas.87.6.2324.

Pettifor, R. A., Perrins, C. M., and McCleery, R. H. (2001). The individual optimization of fitness: Variation in reproductive output, including clutch size, mean nestling mass and offspring recruitment, in manipulated broods of great tits Parus major. *J. Anim. Ecol.* 70, 62–79. doi:10.1046/j.1365-2656.2001.00465.x.

Piersma, T. (2011). Why marathon migrants get away with high metabolic ceilings: Towards an ecology of physiological restraint. *J. Exp. Biol.* 214, 295–302. doi:10.1242/jeb.046748.

Pörtner, H. O., Bock, C., and Mark, F. C. (2017). Oxygen- & capacity-limited thermal tolerance: Bridging ecology & physiology. *J. Exp. Biol.* 220, 2685–2696. doi:10.1242/jeb.134585.

Portugal, S. J., Green, J. A., Halsey, L. G., Arnold, W., Careau, V., Dann, P., et al. (2016). Associations between resting, activity, and daily metabolic rate in free-living endotherms: No universal rule in birds and mammals. *Physiol. Biochem. Zool.* 89, 251–261. doi:10.1086/686322.

R, C. T. (2021). R: A language and environment for statistical computing. Available at: https://www.r-project.org/.

Ricklefs, R. E. (1984). The Optimization of Growth Rate in Altricial Birds. *Ecology* 65, 1602–1616.

Ricklefs, R. E., Konarzewski, M., and Daan, S. (2006). The Relationship between Basal Metabolic Rate and Daily Energy Expenditure in Birds and Mammals. 147, 1047–1071.

Roff, D. (1993). *Evolution Of Life Histories: Theory and Analysis*. Springer Science & Business Media.

Rogowitz, G. L. (1998). Limits to milk flow and energy allocation during lactation of the hispid cotton rat (Sigmodon hispidus). *Physiol. Zool.* 71, 312–320. doi:10.1086/515923.

Ronget, V., Gaillard, J. M., Coulson, T., Garratt, M., Gueyffier, F., Lega, J. C., et al. (2018). Causes and consequences of variation in offspring body mass: meta-analyses in birds and mammals. *Biol. Rev.* 93, 1–27. doi:10.1111/brv.12329.

Rutkowska, J., Cichoń, M., Puerta, M., and Gil, D. (2005). Negative effects of elevated testosterone on female fecundity in zebra finches. *Horm. Behav.* 47, 585–591. doi:10.1016/j.yhbeh.2004.12.006.

Rutkowska, J., Sadowska, E. T., Cichon, M., and Bauchinger, U. (2016). Increased fat catabolism sustains water balance during fasting in zebra finches. 2623–2628. doi:10.1242/jeb.138966.

Sadowska, E. T., Stawski, C., Rudolf, A., Dheyongera, G., Chrązcik, K. M., Baliga-Klimczyk, K., et al. (2015). Evolution of basal metabolic rate in bank voles from a multidirectional selection experiment. *Proc. R. Soc. B Biol. Sci.* 282, 20150025–20150025. doi:10.1098/rspb.2015.0025.

Sadowska, J., Gębczynski, A. K., Lewoc, M., and Konarzewski, M. (2019). Not that hot after all: No limits to heat dissipation in lactating mice selected for high or low BMR. *J. Exp. Biol.* 222. doi:10.1242/jeb.204669.

Salin, K., Auer, S. K., Rudolf, A. M., Anderson, G. J., Cairns, A. G., Mullen, W., et al. (2015). Individuals with higher metabolic rates have lower levels of reactive oxygen species in vivo. *Biol. Lett.* 11, 4–7. doi:10.1098/rsbl.2015.0538.

Salin, K., Villasevil, E. M., Anderson, G. J., Auer, S. K., Selman, C., Hartley, R. C., et al. (2018). Decreased mitochondrial metabolic requirements in fasting animals carry an oxidative cost. *Funct. Ecol.* 32, 2149–2157. doi:10.1111/1365-2435.13125.

Santos, E. S. A., and Nakagawa, S. (2012). The costs of parental care: A meta-analysis of the trade-off between parental effort and survival in birds. *J. Evol. Biol.* 25, 1911–1917. doi:10.1111/j.1420-9101.2012.02569.x.

Sanz, J. J., and Tinbergen, J. M. (1999). Energy expenditure, nestling age, and brood size: An experimental study of parental behavior in the great tit Parus major. *Behav. Ecol.* 10, 598–606. doi:10.1093/beheco/10.5.598.

Schaper, S. V., and Visser, M. E. (2013). Great tits provided with ad libitum food lay larger eggs when exposed to colder temperatures. *J. Avian Biol.* 44, 245–254. doi:10.1111/j.1600-048X.2012.00129.x.

Schifferli, L., Grüebler, M. U., Meijer, H. A. J., Visser, G. H., and Naef-Daenzer, B. (2014). Barn Swallow Hirundo rustica parents work harder when foraging conditions are good.

Ibis (Lond. 1859). 156, 777–787. doi:10.1111/ibi.12186.

Schmidt-Nielsen, K. (1972). *How animals work*. Cambridge University Press.

Scholander, P. F., Hock, R., Walters, V., Irving, L., and Irving4, L. (1950). Adaptation to Cold in Arctic and Tropical Mammals and Birds in Relation to Body Temperature, Insulation, and Basal Metabolic Rate. *Source Biol. Bull.* 99, 259–271. Available at: http://www.jstor.org/stable/1538742.

Schwagmeyer, P. L., and Mock, D. W. (2008). Parental provisioning and offspring fitness: size matters. *Anim. Behav.* 75, 291–298. doi:10.1016/j.anbehav.2007.05.023.

Selman, C., McLaren, J. S., Himanka, M. J., and Speakman, J. R. (2000). Effect of long-term cold exposure on antioxidant enzyme activities in a small mammal. *Free Radic. Biol. Med.* 28, 1279–1285. doi:10.1016/S0891-5849(00)00263-X.

Senar, J. C., and Pascual, J. (1997). Keel and tarsus length may provide a good predictor of avian body size. *Ardea* 85, 269–274.

Sies, H. (1997). Physiological society symposium: Impaired endothelial and smooth muscle cell function in oxidative stress. *Exp. Physiol.* 82, 291–5.

Smith, H. G., Hans, K., Fontell, K., and Ljungstrsm, M. (1988). Feeding frequency and parental division of labour in the double-brooded great tit Parus major Effects of manipulating brood size. 447–453.

Speakman, J. (1993). How should we calculate CO2 production in doubly labeled water studies of animals? *Funct. Ecol.* 7, 746–750.

Speakman, J. R. (1997). *Doubly-labelled water: theory and practice*. London: Chapman and Hall.

Speakman, J. R. (2000). *The Cost of Living: Field Metabolic Rates of Small Mammals.*

Advances in ecological reserch.

Speakman, J. R., Blount, J. D., Bronikowski, A. M., Buffenstein, R., Isaksson, C., Kirkwood, T. B. L., et al. (2015). Oxidative stress and life histories: Unresolved issues and current needs. *Ecol. Evol.* 5, 5745–5757. doi:10.1002/ece3.1790.

Speakman, J. R., Ergon, T., Cavanagh, R., Reid, K., Scantlebury, D. M., and Lambin, X. (2003). Resting and daily energy expenditures of free-living field voles are positively correlated but reflect extrinsic rather than intrinsic effects. *Proc. Natl. Acad. Sci. U. S. A.* 100, 14057–14062. doi:10.1073/pnas.2235671100.

Speakman, J. R., and Garratt, M. (2014). Oxidative stress as a cost of reproduction: Beyond the simplistic trade-off model. *BioEssays* 36, 93–106. doi:10.1002/bies.201300108.

Speakman, J. R., and Król, E. (2010). The heat dissipation limit theory and evolution of life histories in endotherms-time to dispose of the disposable soma theory? *Integr. Comp. Biol.* 50, 793–807. doi:10.1093/icb/icq049.

Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., et al. (2004). Uncoupled and surviving: Individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* 3, 87–95. doi:10.1111/j.1474-9728.2004.00097.x.

Speakman, J. R., and Ward, S. (1998). Infrared thermography : principles and applications. *Zoology* 101, 224–232.

Stearns, S. C. (1992). The evolution of life histories.

Stearns, S. C. (2000). Life history evolution: Successes, limitations, and prospects. *Naturwissenschaften* 87, 476–486. doi:10.1007/s001140050763.

Stier, A., Bize, P., Roussel, D., Schull, Q., Massemin, S., and Criscuolo, F. (2014).

Mitochondrial uncoupling as a regulator of life-history trajectories in birds: An experimental study in the zebra finch. *J. Exp. Biol.* 217, 3579–3589. doi:10.1242/jeb.103945.

Stier, A., Reichert, S., Massemin, S., Bize, P., and Criscuolo, F. (2012). Constraint and cost of oxidative stress on reproduction: Correlative evidence in laboratory mice and review of the literature. *Front. Zool.* 9. doi:10.1186/1742-9994-9-37.

Stier, A., Schull, Q., Bize, P., Lefol, E., Haussmann, M., Roussel, D., et al. (2019). Oxidative stress and mitochondrial responses to stress exposure suggest that king penguins are naturally equipped to resist stress. *Sci. Rep.* 9, 1–12. doi:10.1038/s41598-019-44990-x.

Sudyka, J., Casasole, G., Rutkowska, J., and Cichoń, M. (2016). Elevated reproduction does not affect telomere dynamics and oxidative stress. *Behav. Ecol. Sociobiol.* 70, 2223–2233. doi:10.1007/s00265-016-2226-8.

Svensson, L. (1992). *Identification Guide to European Passerines*. 4th Editio. , ed. B. T. for Ornithology Stockholm.

Tapper, S., Nocera, J. J., and Burness, G. (2020a). Experimental evidence that hyperthermia limits offspring provisioning in a temperate-breeding bird: Hyperthermia influences parental care. *R. Soc. Open Sci.* 7. doi:10.1098/rsos.201589.

Tapper, S., Nocera, J. J., and Burness, G. (2020b). Heat dissipation capacity influences reproductive performance in an aerial insectivore. *J. Exp. Biol.* 223. doi:10.1242/jeb.222232.

Thurber, C., Dugas, L. R., Ocobock, C., Carlson, B., Speakman, J. R., and Pontzer, H. (2019). Extreme events reveal an alimentary limit on sustained maximal human energy expenditure. *Sci. Adv.* 5, 1–9. doi:10.1126/sciadv.aaw0341.

Tieleman, B. I., Dijkstra, T. H., Klasing, K. C., Visser, G. H., and Williams, J. B. (2008).

Effects of experimentally increased costs of activity during reproduction on parental investment and self-maintenance in tropical house wrens. *Behav. Ecol.* 19, 949–959. doi:10.1093/beheco/arn051.

Tinbergen, J. M., and Verhulst, S. (2000). A fixed energetic ceiling to parental effort in the great tit? *J. Anim. Ecol.* 69, 323–334. doi:10.1046/j.1365-2656.2000.00395.x.

Tsahar, E., Arad, Z., Izhaki, I., and Guglielmo, C. G. (2006). The relationship between uric acid and its oxidative product allantoin: A potential indicator for the evaluation of oxidative stress in birds. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 176, 653–661. doi:10.1007/s00360-006-0088-5.

Tucker, R., and Noakes, T. D. (2009). The physiological regulation of pacing strategy during exercise: A critical review. *Br. J. Sports Med.* 43, 1–9. doi:10.1136/bjsm.2009.057562.

Ummenhofer, C. C., and Meehl, G. A. (2017). Extreme weather and climate events with ecological relevance: A review. *Philos. Trans. R. Soc. B Biol. Sci.* 372. doi:10.1098/rstb.2016.0135.

Vaanholt, L. M., Speakman, J. R., Garland, T., Lobley, G. E., and Visser, G. H. (2008). Protein synthesis and antioxidant capacity in aging mice: Effects of long-term voluntary exercise. *Physiol. Biochem. Zool.* 81, 148–157. doi:10.1086/525289.

van de Crommenacker, J., Richardson, D. S., Koltz, A. M., Hutchings, K., and Komdeur, J. (2012). Parasitic infection and oxidative status are associated and vary with breeding activity in the Seychelles warbler. *Proc. R. Soc. B Biol. Sci.* 279, 1466–1476. doi:10.1098/rspb.2011.1865.

Vézina, F., Speakman, J. R., and Williams, T. D. (2006). INDIVIDUALLY VARIABLE ENERGY MANAGEMENT STRATEGIES IN RELATION TO ENERGETIC COSTS OF EGG PRODUCTION. *Ecology* 87, 2447–2458.

Viblanc, V. A., Schull, Q., Roth, J. D., Rabdeau, J., Saraux, C., Uhlrich, P., et al. (2018). Maternal oxidative stress and reproduction: Testing the constraint, cost and shielding hypotheses in a wild mammal. *Funct. Ecol.* 32, 722–735. doi:10.1111/1365-2435.13032.

Visser, M. E., Van Dooremalen, C., Tomotani, B. M., Bushuev, A., Meijer, H. A. J., Te Marvelde, L., et al. (2019). Short-term, but not long-term, increased daytime workload leads to decreased night-time energetics in a free-living song bird. *J. Exp. Biol.* 222. doi:10.1242/jeb.199513.

Ward, S., Rayner, J. M. V., Möller, U., Jackson, D. M., Nachtigall, W., and Speakman, J. R. (1999). Heat transfer from starlings Sturnus vulgaris during flight. *J. Exp. Biol.* 202, 1589–1602. doi:10.1242/jeb.202.12.1589.

Wegmann, M., Voegeli, B., and Richner, H. (2015). Physiological responses to increased brood size and ectoparasite infestation: Adult great tits favour self-maintenance. *Physiol. Behav.* 141, 127–134. doi:10.1016/j.physbeh.2015.01.017.

Weiner, J. (1992). Physiological Limits to Sustainable Energy Budgets in Birds and Mammals. 7, 1–5. Available at: file:///Users/melindaconners/Dropbox/Papers2/Articles/2003/Unknown/2003-135.pdf%5Cnfile:///Users/melindaconners/Documents/PDFs/Papers2/Articles/2003/Unknown/2003-135.pdf.

Welcker, J., Speakman, J. R., Elliott, K. H., Hatch, S. A., and Kitaysky, A. S. (2015). Resting and daily energy expenditures during reproduction are adjusted in opposite directions in free-living birds. *Funct. Ecol.* 29, 250–258. doi:10.1111/1365-2435.12321.

West, G. B., Brown, J. H., and Enquist, B. J. (1999). The fourth dimension of life: Fractal geometry and allometric scaling of organisms. *Science (80-.).* 284, 1677–1679. doi:10.1126/science.284.5420.1677.

Wiersma, P., Selman, C., Speakman, J. R., and Verhulst, S. (2004). Birds sacrifice oxidative protection for reproduction. *Proc. R. Soc. B Biol. Sci.* 271, 360–363. doi:10.1098/rsbl.2004.0171.

Wiersma, P., and Tinbergen, J. M. (2003). No nocturnal energetic savings in response to hard work in free-living great tits. *Netherlands J. Zool.* 52, 263–279. doi:10.1163/156854203764817715.

Williams, T. D., and Cooch, E. G. (1996). Egg Size , Temperature and Laying Sequence: Why do Snow Geese Lay Big Eggs When it's cold? *Funct. Ecol.* 10, 112–118.

Williams, T. D., and Vézina, F. (2001). "REPRODUCTIVE ENERGY EXPENDITURE, INTRASPECIFIC VARIATION AND FITNESS IN BIRDS," in *Current Ornithology*.

Williamson, K., Gilbert, L., Rutstein, A. N., Pariser, E. C., and Graves, J. A. (2008). Within-year differences in reproductive investment in laboratory zebra finches (Taeniopygia guttata), an opportunistically breeding bird. *Naturwissenschaften* 95, 1143–1148. doi:10.1007/s00114-008-0436-2.

Xu, Y. C., Yang, D. B., Speakman, J. R., and Wang, D. H. (2014). Oxidative stress in response to natural and experimentally elevated reproductive effort is tissue dependent. *Funct. Ecol.* 28, 402–410. doi:10.1111/1365-2435.12168.

Yang, D. B., Xu, Y. C., Wang, D. H., and Speakman, J. R. (2013). Effects of reproduction on immuno-suppression and oxidative damage, and hence support or otherwise for their roles as mechanisms underpinning life history trade-offs, are tissue and assay dependent. *J. Exp. Biol.* 216, 4242–4250. doi:10.1242/jeb.092049.

Yap, K. N., Kim, O. R., Harris, K. C., and Williams, T. D. (2017). Physiological effects of increased foraging effort in a small passerine. *J. Exp. Biol.* 220, 4282–4291. doi:10.1242/jeb.160812.

Yap, K. N., Powers, D. R., Vermette, M. L., Tsai, O. H. I., and Williams, T. D. (2021). Physiological adjustments to high foraging effort negatively affect fecundity but not final reproductive output in captive zebra finches. *J. Exp. Biol.* 224, 1–10. doi:10.1242/JEB.235820.

Zagkle, E., Grosiak, M., Bauchinger, U., and Sadowska, E. T. (2020). Rest-Phase Hypothermia Reveals a Link Between Aging and Oxidative Stress: A Novel Hypothesis. *Front. Physiol.* 11, 1–12. doi:10.3389/fphys.2020.575060.

Zann, R. (1996). *The Zebra Finch: A Synthesis of Field and Laboratory Studies*. New York, USA: Oxford University Press Available at: https://books.google.be/books/about/The_Zebra_Finch.html?id=5KO6cZH0WbEC&redir_esc=y.

Zera, A. J., and Harshman, L. G. (2007). The Physiology of Life History Trade-Offs in Animals Anthony J . Zera and Lawrence G . Harshman. *Ecology* 32, 95–126.

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Figure 22. A) Female body mass (g) from the hatching date (day 0) until offspring development (day 35) between cold (blue boxplots) and warm (red boxplots) ambient temperatures, and between feather-clipped (open boxplots) and unclipped (filled boxplots). B) Nestling development under cold (14 °C) and warm (25 °C) conditions and those raised from females in which they were feather-clipped (open points/boxplots) and unclipped (filled points/boxplots). Total 153 nestlings on day 0 from total 46 nests (cold; n = 100 and warm; n = 53), total 142 nestlings from 43 nets on day 6 which feather-clip manipulation took place (cold and feather-clipped; n = 50, cold and unclipped; n = 45 and warm and feather-clipped; n = 22, warm and unclipped; n = 25). Total 114 nestlings from 42 nests on day 35 (cold and feather-clipped; n = 44, cold and unclipped; n = 33 and warm and feather-clipped; n = 18, warm and unclipped; n = 19). (same Figure as Supplementary Figure 5 from Zagkle et al., 2022, an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY)).....92

PUBLICATIONS AND COMMUNICATION ASSOCIATED WITH THE DOCTORAL DISSERTATION

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Conferences

Zagkle, E., Grosiak, M., Kunysz, S., Cichoń, M., and Bauchinger, U., Sadowska, E. T. (2018). "Ageing and thermal conductance in zebra finches (*Taenopygia guttata*)". 27th International Ornithological Congress, August 2018, Vancouver, Canada. **ORAL TALK**

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Bryla, A., **Zagkle, E.**, Sadowska, E. T., Cichoń, M., and Bauchinger, U. (2021). "Body temperature as an indicator and driver of costs associated with avian humoral response". 21st Virtual Annual Meeting of the Society of Integrative and & Comparative of Biology.

Zagkle, E., Bauchinger, U., Sadowska, E. T. (2018). “Age-specific differences in thermoregulation in zebra finches”. 6th Polish Evolutionary Conference, Warsaw, Poland. **PRIZE FOR THE BEST POSTER PRESENTATION**

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