Fitness consequences of physiological responses to environmental variation in wild great tits

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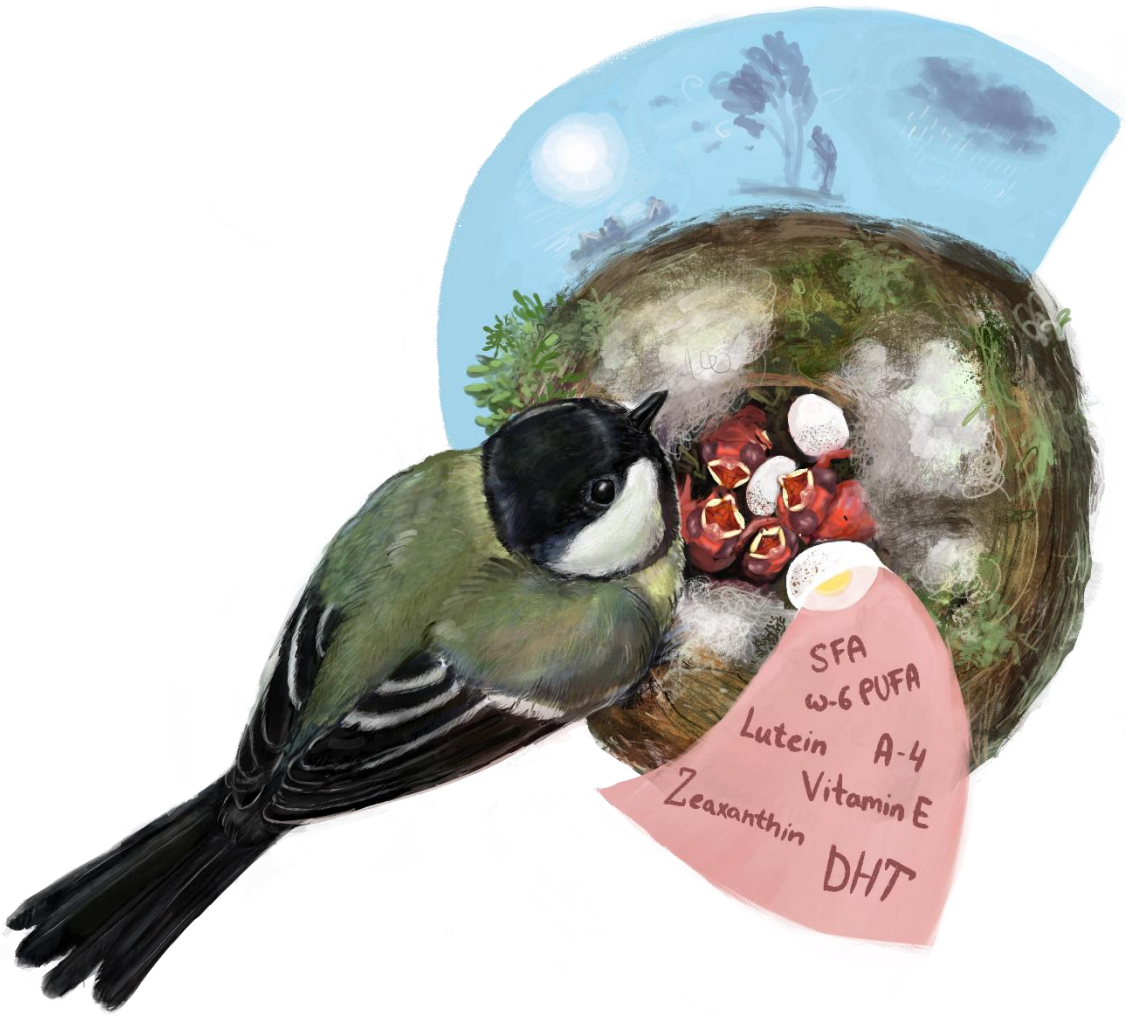

Lucía Mentesana

Drawing by Alena Lemazina

A mi familia,

por su amor y apoyo incondicional…

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Summary

Environments change continuously. Fluctuations in temperature, rainfall, and food availability, can be predictable on the long term, but unpredictable within short time frames. Additionally, fluctuations in the environment can now occur unpredictably and with increasing frequency and duration because of human activities. Changes in an organism's environment can be challenging because they destabilize its homeostatic processes. Understanding how organisms respond to these homeostatic disturbances, and if and how they can cope with them has become critically important and urgently needed in the light of climate change and increasing human disturbance.

Physiological signals serve to integrate environmental and genomic information, and transmit that information within the organism to mediate a phenotypic response. Physiological traits, which refer to aspects of organism functioning, like nutrition, metabolism, thermal relationships, endocrine responses, and changes in immune parameters are important mechanisms to cope with fluctuating environments. If these traits directly affect an organism's fitness and have transgenerational effects that also affect the phenotype and survival of its offspring, then they are potentially important drivers for evolutionary processes in response to rapidly changing environments.

The general aim of my thesis was to determine the fitness consequences of physiological responses to environmental variation. For this, I studied how the physiological response of parents to environmental conditions relates to patterns of reproductive investment, reproductive success and offspring fitness in a wild population of great tits (*Parus major*).

I first studied how the physiological response of parents to environmental conditions relates to their reproductive success (chapter 2). I tested the hypothesis that physiological traits covary and predict fitness primarily under challenging environmental conditions. I used data on six physiological traits (i.e., glucocorticoids: baseline and stress-induced concentrations, oxidative status: pro-oxidants, dietary and enzymatic antioxidant concentrations, and body condition) and fitness proxies (i.e., fledgling number and mass) collected over two breeding seasons that significantly differed in environmental conditions. Chapter 2 shows that physiological traits do not covary but they predict, in a sex-specific way, reproductive success when environmental conditions are challenging. In the year with low ambient temperature and high cumulated rainfall, females in a better oxidative condition produced more and heavier fledglings, while males with high body condition fledged more offspring. In males, but not in females, high baseline and low stress-induced glucocorticoid

concentrations were also positively and negatively related to high fledgling mass. Chapter 2 suggests that glucocorticoids, oxidative state and body condition might be important mediators to successfully cope with challenging environmental circumstances.

The way in which mothers cope with environmental changes can affect their reproductive investment, by altering the resources mothers pass on to their developing embryos. In egg laying species, females deposit hormones, nutrients and immune components into their eggs. In birds, the patterns of yolk deposition of these components along the laying sequence vary across and within species, but the processes that shape female yolk deposition are not yet fully understood. In chapter 3, I used a statistical approach based on repeated measures to study female yolk deposition along the laying sequence at both the population and among-female levels. I used data on the concentrations of 11 yolk components including steroid hormones, antioxidants, and fatty acids, which I determined in the eggs of 11 entire clutches collected during one breeding season. Chapter 3 shows that variation in yolk deposition at a population level is underpinned by different individual patterns, and that these individual patterns may be shaped by both genetic and environmental components. It also confirms that the method of analyzing the fourth egg from a nest is a suitable way to estimate clutch-level yolk composition in studies of wild populations. Further, chapter 3 shows that mothers concomitantly secret steroid hormones, antioxidants and fatty acids into their egg yolks, suggesting that maternal substances work interactively within an egg to influence the phenotype of the offspring.

In chapter 4, I built on the knowledge obtained in the previous chapter to test for interactive effects of yolk components on offspring phenotypic traits (i.e., oxidative state and body condition) and fitness proxies (i.e., hatchling number, fledgling number, mass and tarsus). I used data on concentrations of 31 yolk components measured in the fourth egg from 69 nests collected over two breeding seasons. Chapter 4 shows that offspring phenotypes relate to the interactions among the yolk components that females deposit into their eggs. This chapter also provides the first evidence for a relationship between yolk fatty acids and hatchling and fledgling number in a wild population; thus, suggesting that these yolk components, which are strongly influenced by the quantity and quality of the food consumed by mothers during egg laying, can greatly influence an individual's fitness. In summary, chapter 3 and 4 show that the way mothers cope with environmental changes affects the resources they transfer to their eggs, which interact to shape phenotypic and fitness traits in the offspring.

With this thesis I thus contributed empirically to the study of how changes in physiological traits due to environmental variation relate to fitness and phenotypic responses. My thesis shows that birds from a free-living population can successfully cope with unpredictable environmental disturbances during the breeding season, such as low temperatures and high cumulated rainfall, and suggests that glucocorticoids, oxidative state and body condition are important mediators to do so. However, female reproductive success might be negatively affected via transgenerational effects on offspring fitness if fluctuations in environmental conditions cause a decrease in food supply, and therefore a decrease in essential yolk components transferred by the mothers into the eggs. Overall, my thesis shows that the interactions between parents and offspring fitness are an important point to include in studies aiming to understand if and how organisms cope with environmental changes.

Zusammenfassung

Die Umwelt verändert sich stetig. Langfristig Änderungen in Temperatur, Niederschlag und Futterverfügbarkeit sind vorhersehbar, jedoch können sie kurzfristig unvorhersehbar fluktuieren. Diese kurzfristigen Fluktuationen werden durch menschliche Aktivitäten verstärkt und treten nun öfters und über längere Zeiträume auf. Solche Veränderungen destabilisieren homöostatische Prozesse und stellen daher eine Herausforderung für Organismen dar. In Bezug auf den Klimawandel und zunehmende menschliche Störungen ist es dringend nötig zu verstehen, wie Organismen auf solche homöostatischen Herausforderungen reagieren, und ob und in welcher Weise sie diese bewältigen.

Physiologische Signale integrieren genetische und ökologische Informationen und leiten diese innerhalb des Organismus weiter, um eine phänotypische Antwort zu vermitteln. Physiologische Merkmale, die sich auf Aspekte der Funktionsweise des Organismus beziehen wie auf Ernährung, Metabolismus, thermische Verhältnisse, endokrine Antworten und Änderungen in Immunparametern, sind notwenige Mechanismen, welche die Anpassung an fluktuierende Umweltbedingungen ermöglichen. Wenn diese Mechanismen die Fitness eines Organismus direkt beeinflussen, und außerdem auch generationsübergreifende Effekte auf den Phänotyp und die Überlebenschancen des Nachwuchses haben, dann sind dies potenziell wichtigen Antriebe für evolutionäre Prozesse in sich schnell verändernden Lebensräumen.

Das allgemeine Ziel meiner Doktorarbeit war die Auswirkungen von physiologischen Antworten in Bezug auf unterschiedliche Umweltbedingungen auf die Fitness eines Organismus zu untersuchen. Dazu habe ich in einer freilebenden Kohlmeisen-Population (*Parus major*) dahingehend untersucht, wie die physiologischen Antworten der Eltern auf unterschiedliche Umweltbedingungen ihren Fortpflanzungsaufwand und Fortpflanzungserfolg beeinflussen, und auch die Fitness ihrer Nachkommen.

Ich untersuchte zunächst, wie die physiologische Reaktion der Elterntiere auf die jeweiligen Umweltbedingungen deren Fortpflanzungserfolg beeinflusst (Kapitel 2). Dafür habe ich die Hypothese getestet, dass physiologische Merkmale die Fitness vor allem unter herausfordernden/schwierigen/anspruchsvollen Bedingungen vorhersagen Dafür habe ich Daten aus zwei Brutsaisons verwendet, die sich hinsichtlich ihrer Umweltbedingungen erheblich unterschieden haben, und sechs physiologische Merkmale (Glukokortikoide: Basis- und

Stressinduzierte Konzentrationen, oxidativer Status: Pro-Oxidantien, Nahrungs- und enzymatische Antioxidantienkonzentrationen, und körperlicher Zustand) und Fitnessparameter (Anzahl und Gewicht der flüggen Küken) verglichen. Die Ergebnisse aus Kapitel 2 weisen darauf hin, dass die Beziehungen zwischen physiologischen Merkmalen und Fitnessparametern nur in dem Jahr mit klimatisch herausfordernden Bedingungen nachweisbar waren, und dass diese Beziehungen geschlechtsspezifisch sind, da sie sich bei Männchen und Weibchen unterschieden. Im Jahr mit niedrigen Temperaturen und hoher Niederschlagsmenge hatten Weibchen in einem besseren oxidativen Zustand mehr und schwerere flügge Küken, wohingegen bei den Männchen ein Anstieg in der Zahl flügger Küken mit einem guten körperlichen Zustand korreliert war. Außerdem waren bei Männchen, nicht aber bei den Weibchen, hohe Basiswerte in den Glukokortikoidkonzentrationen und gleichzeitig geringe stressinduzierte Glukokortikoidreaktionen positiv mit dem Gewicht der flüggen Küken assoziiert. Kapitel 2 legt nahe, dass Glukokortikoide, der oxidative Status und die Körperliche Verfassung wichtige Vermittler sein könnten, um schwierigen Umweltbedingungen erfolgreich zu bewältigen.

Wie Mütter auf Änderungen der Umweltbedingungen reagieren kann ihre Fortpflanzungsaufwand beeinflussen, indem sie die Ressourcen verändert, die sie an ihre sich entwickelnden Embryonen weitergeben. Bei eierlegenden Arten verbringen Weibchen Hormone, Nährstoffe und Immunkomponenten in ihre Eier. Bei Vögeln ist bekannt, dass die Zusammensetzung dieser Komponenten der Dotterdeposition sich über die Legereihenfolge innerhalb sowie zwischen Arten unterscheiden. Die diesem zugrundeliegenden Prozesse sind jedoch bisher wenig bekannt. In Kapitel 3 habe ich einen statistischen Ansatz verwendet, der auf wiederholten Messungen basiert, um die Zusammensetzung der Dotterdeposition über die Legereihenfolge für die Population und zwischen den Weibchen zu bestimmen. Dafür habe ich die Konzentrationen von elf Dotterkomponenten, einschließlich Steroidhormonen, Antioxidantien und Fettsäuren, in den Eiern aus elf kompletten Gelegen einer Saison gemessen. Kapitel 3 zeigt, dass die Variation in der Dotterdeposition über die Legereihenfolge durch die individuellen Muster der einzelnen Weibchen hervorgerufen wird, und dass diese individuellen Muster vermutlich durch genetische und umweltbedingte Faktoren geformt werden. Darüber hinaus konnte ich mit dieser Studie zeigen, dass die Analyse der Dotterbestandteile im vierten Ei eines Geleges repräsentativ für das ganze Gelege ist, und dies damit eine geeignete Methode ist, um die Dotterzusammensetzung eines ganzen Geleges in freilebenden Populationen zu bestimmen. Außerdem zeigt Kapitel 3, dass

Muttervögel gleichzeitig Steroidhormone, Antioxidantien und Fettsäuren in ihre Eidotter abscheiden und dass die Interaktion dieser mütterlichen Ressourcen den Phänotyp des Kükens beeinflussen.

Das vierte Kapitel baut auf den im vorherigen Kapitel gewonnenen Erkenntnissen auf, um die Interaktionen zwischen den Dotterkomponenten zu testen, und ihre Beziehungen zu phänotypischen Merkmalen der Nachkommen (d.h. Oxidationszustand und körperlicher Zustand) und Fitness (d.h. Anzahl der geschlüpften Jungtiere, Anzahl der Jungtiere sowie Gewicht und Tarsuslänge) zu untersuchen. Dafür habe ich 31 Dotterkomponenten im jeweils vierten Ei von 69 verschiedenen Gelegen in zwei aufeinanderfolgenden Brutsaisonen gemessen. In Kapitel 4 zeige ich, dass der Phänotyp der Küken von den Interaktionen der Dotterkomponenten, welche die Weibchen in die Eier verbringen, bestimmt wird. Außerdem zeige ich zum ersten Mal in diesem Kapitel, dass die Konzentration der verschiedenen Fettsäuren im Dotter durch die Quantität und Qualität des Futters, dass der Muttervogel während der Eiablage zu sich nimmt, bestimmt wird, und dass diese Fettsäuren einen Zusammenhang mit der Anzahl der geschlüpften und flüggen Küken haben. Dies deutet darauf hin, dass die Fitness der Muttervögel auch davon bedingt wird, wie die jeweiligen Umweltbedingungen die Verfügbarkeit von Nahrungsressourcen beeinflussen, da dies sich auf die phänotypischen und Fitness-Merkmale der Küken auswirkt.

Mit meiner Doktorarbeit habe ich einen empirischen Beitrag zu der Erforschung der Zusammenhänge zwischen Umweltveränderungen, physiologischen Merkmalen, und phänotypischen Reaktionen. Meine Arbeit belegt, dass wildlebende Vögel unvorhersehbare, herausfordernde Umweltbedingungen, wie hohe Niederschlagsmengen und niedrige Temperaturen, während der Brutzeit erfolgreich bewältigen können, und dass Glukokortikoide, oxidativer Status und körperlicher Zustand grundlegende vermittelnde Faktoren dafür sind. Jedoch können generationsübergreifende Effekte den Fortpflanzungserfolg von Weibchen negativ beeinflussen, wenn fluktuierende Umweltbedingungen eine Abnahme in der Nahrungsverfügbarkeit bedingen, und dadurch die Zusammensetzung der Dotterkomponenten negativ beeinflusst wird. Zusammenfassend zeigt meine Doktorarbeit, dass die Einbeziehung der Interaktion zwischen Eltern und der Fitness der Nachkommen ein wichtiger Punkt für zukünftige Forschung ist, wenn verstanden werden soll wie Organismen auf Umweltveränderungen reagieren.

1. General introduction

1.1. Phenotypic variation in response to environmental changes

Environments change continuously. Environmental changes can occur due to fluctuations in abiotic factors such as temperature, barometric pressure, precipitation, and food availability, and in biotic factors like predation pressure, social interactions and social status. These changes can be predictable over long-term periods, but unpredictable within short time frames. Additionally, fluctuations in the environment can now occur unpredictably and with increasing frequency, duration and intensity because of human activities (Wingfield 2015).

Variation in the environment can be challenging for an organism by destabilizing its homeostatic processes. To avoid this, organisms change their morphology, behavior and/or physiology in response to environmental conditions (e.g., Meyers & Bull 2002; Whitman & Agrawal 2009). However, not all organisms respond in a similar way: environmental changes create phenotypic variation. Environmentally induced phenotypic variation constitutes an important source of variation in natural populations, with potential fitness and evolutionary consequences (Pigliucci 2005, Whitman & Agrawal 2009). That is, phenotypic variation determines the performance of organisms in response to environmental stimuli and this performance can determine the fitness of alternative genotypes, thus determining the frequency of genotypes carried into the next generation.

Phenotypic variation in response to environmental changes can also lead to transgenerational effects, known as 'parental effects'. The way parents respond to environmental changes can influence their offspring phenotype through the transmission of factors other than DNA (Bernardo 1996, Mousseau & Fox, 1998, Agrawal et al. 1999; Meyers & Bull 2002). Parental effects are present in a wide range of taxa (i.e., plants, insects, vertebrates) and contribute substantially to phenotypic variation within populations (Badyaev 2008, Kuijper & Hoyle 2015, Moore et al. 2019, Yin et al. 2019). Parental effects can derive from the nutritional and physiological status of the parent or from further environmental changes generated by the parents. Examples are neriid flies (*Telostylinus angusticollis*), in which offspring body size and head elongation are mediated by the amount of proteins and carbohydrates present in maternal and paternal diets (Bonduriansky et al. 2016), or dung beetles (*Onthophagus taurus*), in which maternal and paternal provisioning affects the body size and horn size of offspring produced (Hunt & Simmons 2000).

Despite the profound interest in phenotypic variation within the field of evolutionary biology and ecology, a central question remains: what are the mechanisms underlying phenotypic variation among individuals of a population in response to environmental stimuli?

1.2. Physiological traits as key mechanisms underlying phenotypic variation

Physiological traits usually refer to aspects of organism functioning, like nutrition, metabolism, thermal relationships, endocrine responses and changes in immune parameters (Ricklefs & Wikelski 2002).

Because they mediate the relationship of the organism to its environment, physiological traits have been proposed as key mechanisms underlying the diversification of life histories traits among individuals (Zera & Harshman 2001, Ricklefs & Wikelski 2002). Their role in mediating phenotypic adjustments has been demonstrated in a wide variety of taxa, such as plants, invertebrates and vertebrates (e.g., Emlen & Allen 2004; Hulbert 2005; Hau 2007; Hulbert et al. 2007; Whitman & Agrawal 2009; Hau et al. 2010; Hofman & Todgham 2010; Stillwell et al. 2010; Romero & Wingfield 2016; Goymann et al. 2019).

Physiological traits might be important targets for selection. Variation in physiological traits can influence reproductive success and survival (reviewed by e.g., Metcalfe & Monaghan 2001; Møller & Saino 2004; Hulbert et al. 2007, Costantini 2008; Monaghan et al. 2009; Costantini 2014; Hau & Goymman 2015; Blount et al. 2016; Hau et al. 2016; Twinning et al. 2016). For example, cellular oxidative stress can directly decrease the reproductive success of males and females by lowering sperm, oocyte and embryo quality (e.g., Agarwal et al. 2006; Rojas Mora et al. 2017); or poor nutritional condition can shape the lifespan of an individual (e.g., Metcalfe & Monaghan 2001). Physiological traits can also indirectly influence reproductive success and survival through the modification of fitness-relevant phenotypes (e.g., variation in testosterone and corticosterone concentrations covary negatively and positively with parental care, a behavior that is strongly related to reproductive success; Hau 2007; Hau & Goymann 2015, Goymann & Davila 2017). Physiological traits are therefore important to build our understanding of the diversification of phenotypic responses in an organism to cope with novel or changing environments. However, identifying general patterns in how physiological parameters vary in response to environmental conditions, and how these changes relate to phenotypic responses and to fitness has proven

difficult. Substantial variation exists across studies in the type of physiological trait and the direction of its relationships with phenotypic and fitness traits. For instance, individuals exposed to the same environmental stimuli (e.g., food availability) show different physiological responses (e.g., corticosterone concentrations; Lendvai et al. 2014); individuals in a similar physiological condition (e.g., testosterone concentrations) do not always relate in the same way with phenotypic traits (e.g., offspring-feeding rates; Goymann & Davila, 2017); natural variation in physiological traits (e.g., immune system) does not always relate to fitness (e.g., survival; Møller & Saino 2004).

1.3. Moving beyond single-measurements to study the consequences of physiological responses to environmental variation

Studies measuring the relationships between physiological traits and/or studies using statistical methods to decompose phenotypic variation into different hierarchical levels may help address the aforementioned questions.

1.3.1. Physiological traits interact with each other

Researchers testing the phenotypic and fitness consequences of a physiological trait in response to environmental changes commonly measure one physiological trait at a time. This approach has been pivotal to study the presence, intensity and directionality of natural selection on physiological traits in wild populations; however, physiological traits can interact with each other (e.g., Ketterson et al. 2009, Cohen et al. 2012). Environmental changes can induce manifold changes altering suites of independent and interconnected physiological traits, potentially explaining the mixed results sometimes found in studies.

Identifying correlations among physiological components is important. They indicate that the joint action of several physiological parameters, and not single traits, can be responsible for restoring homeostasis and performance in response to environmental changes. The presence of correlations between physiological traits also provides information on how to study physiological traits. If physiological traits are correlated, the apparent phenotypic and fitness consequences of variation in the studied trait might reflect selection acting on variation in the other factor or in the correlation between the two of them. From an evolutionary perspective, the presence of correlations can indicate suites of physiological parameters favored by selection or constraints on the independent evolution of physiological traits (e.g., Pigliucci 2003; Duckworth 2006, Dingemanse et al. 2010).

1.3.2. Phenotypic responses differ among individuals: a reaction norm approach

The population-level response to the environment and the levels of phenotypic variance observed under differing environmental conditions are affected by the way individuals respond to the environment. That is, within a population each individual may respond to its environment in its own particular way. Importantly, the nature of individual response cannot be necessarily inferred from a population analysis (Nussey et al. 2007, Dingemanse et al. 2010, Westneat et al. 2015). Under a similar population-level response (Figure 1.1a), individuals can differ from each other in their mean phenotypic response (Figure 1.1b) and in the degree of change towards an environmental change (Figure 1.1c), and these aspects can sometimes be considered complementary aspects of an individual phenotype (Figure 1.1d).

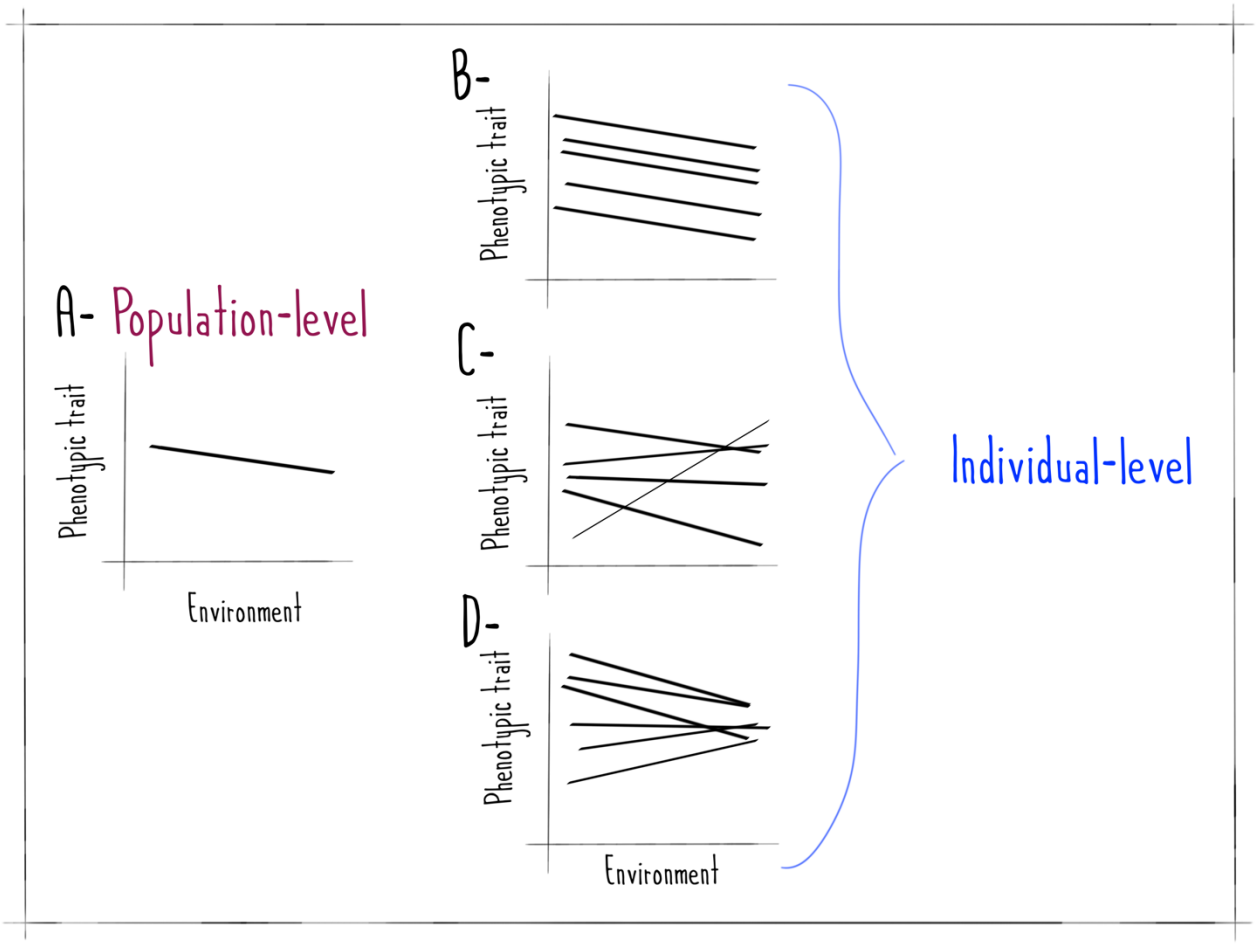

Figure 1.1: Graphical representation of phenotypic variation at the (a) population- and individual level. A population-level response to the environment depends on individual-level variation. Individuals can differ from each other (b) in the mean phenotypic response, (c) in the slope of change along the environmental gradient, or (d) in the covariation between both parameters. Graph adapted from Nussey et al. (2007).

Natural selection acts on the level of the individual, and therefore is a key level to study if we aim to understand how an organism copes with environmental changes. However, how important is it that individuals differ in their mean response to environmental changes? Is this response more, less or equally important than the degree of change? Or is it the combination between both traits (i.e., the mean response *and* the degree of change) that determine the way an individual cope with changes?

Reaction norms are powerful tools to answer these questions. In particular, reaction norms incorporate how animals respond on average and their degree of change over a gradient into a single framework, specifying the precise form of the relationship between phenotypic response and environmental conditions (e.g., Williams 2008; Nussey et al. 2007; Dingemanse et al. 2010). Reaction norms can be quantified by using linear mixed effect models (also known as 'random regression models', Dingemanse et al. 2010). The use of this statistical technique allows us to partition the phenotypic response to gain knowledge on how consistent an individual's phenotypic trait is over time (i.e., the mean phenotypic response), how fast an individual can change its phenotypic response to given environmental gradients (i.e., the slope of change of a phenotypic trait), and how the mean phenotypic response and slope are related. Further, by studying reaction norms using linear mixed effect models we can gain information on potential evolutionary processes shaping phenotypic variation. From the average reaction norm intercept we can calculate repeatability estimates, which can provide insight into the heritable nature of the trait and their potential response to selection (Boake 1989; but see Dohm 2002). Also, if the mean phenotypic response and the slope of change are correlated, and the phenotypic response is related to fitness, this suggests that selection could be acting on the mean phenotypic response, on the slope and/or on the correlation between the two traits.

The concept of reaction norms and the use of linear mixed effect models to quantify them is commonly used in several fields of research such as behavioral ecology and quantitative genetics, among others (Nussey et al. 2007, Dingemanse et al. 2010, Dingemanse & Wolf 2012, Araya-Ajoy et al. 2015, Araya-Ajoy & Dingemanse 2016). Within the field of evolutionary physiology, there have been repeated calls for integrating both population and individual levels of variation (Williams 2008, Hau & Goymann 2015; Hau et al. 2016). However, studies applying a reaction norm approach together with linear mixed effect models in physiology are rare (but see Hsu et al.

2019); probably because it requires a relatively large database of the same individuals sampled repeatedly over an environmental gradient (e.g., Martin et al. 2011; van de Pol 2012).

1.4. How do individuals cope with environmental variation? Glucocorticoids and oxidative status as candidate physiological systems

At present, two major physiological systems are being discussed as candidate mediators of the link among environmental changes, phenotypic variation and fitness: the endocrine and the redox systems.

Among endocrine signals, glucocorticoid hormones have emerged as systemic mediators involved in allowing an organism to cope with environmental challenges because they serve diverse functions to maintain homeostasis, particularly in energy balance, of an organism depending on its needs (McEwen & Wingfield 2003; Landys et al. 2006; Romero et al. 2009; Crespi et al. 2013; Monaghan & Spencer 2014; Hau & Goymann 2015; Hau et al. 2016; Romero & Wingfield 2016). Glucocorticoids, such as cortisol (primarily present in fish and most mammals except for rodents) and corticosterone (present in reptiles, amphibians, birds and rodents), are hormones secreted by the hypothalamic-pituitary-adrenal (HPA) axis which is a major endocrine system in vertebrates (Schmidt & Soma 2008; Romero & Wingfield 2016). Glucocorticoids are typically measured in two contexts (Figure 1.2). First, the baseline activity of the HPA axis is related to an organism's energetic state because glucocorticoids play an important role in regulating circulating levels of glucose (McEwen & Wingfield 2003; Landys et al. 2006). This can occur through different mechanisms such as modulating glucose availability in a tissue-dependent manner, stimulating appetite, and increasing foraging and locomotor activities. Hence, baseline glucocorticoid concentrations are expected to correspond to variation in energetic demands under predictable changes in the environment such as different life history-stages, daily and seasonal activity patterns. Second, glucocorticoids can also coordinate the physiological and behavioral responses to unpredictable environmental changes and/or perceived challenges (Sapolsky et al. 2000; Romero 2004; Hau et al. 2016). This stress-induced situation, which is characterized by a large increase in glucocorticoid concentrations within 2-3 minutes of being exposed to unpredictable stimuli (Romero & Reed 2005), switches the physiology of the animal into an 'emergency state' where survival is prioritized over other less essential activities (e.g., reproduction). In this second case, glucocorticoids facilitate and augment sympathetic interactions (i.e., augment cardiovascular

activation), and act together with other hormones (i.e., catecholamines and glucagon) to elevate glucose concentrations by stimulating glycogenolysis (i.e., breakdown of glycogen to make glucose available) and gluconeogenesis (i.e., synthesis of glucose from non-carbohydrate sources; reviewed in Romero & Wingfield 2016). Within hours after a stress-induced increase, elevated glucocorticoid concentrations return to baseline via negative feedback (McEwen & Wingfield 2003; Romero et al. 2009; Hau et al. 2016).

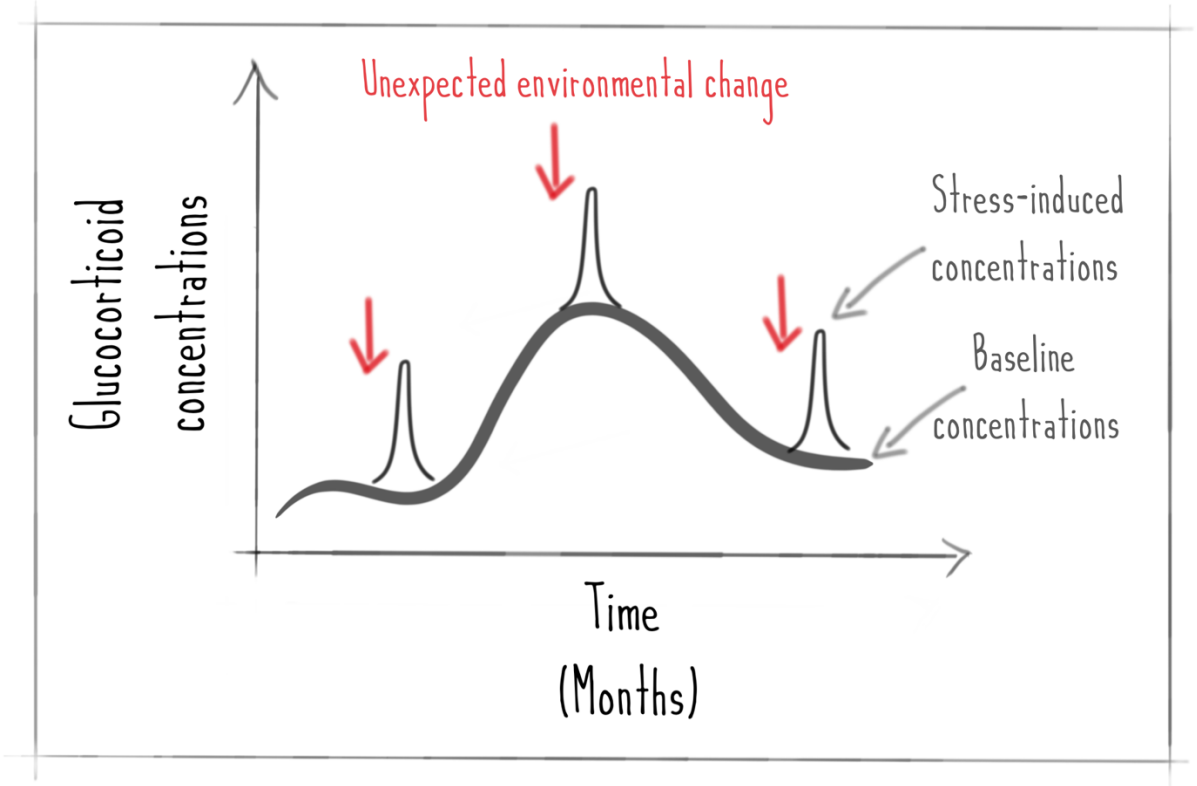

Figure 1.2: Graphical model of the modulation of glucocorticoid concentrations over time. Under predictable changes in the environment like different life-history stages, daily and seasonal activity patterns, baseline concentrations fluctuate to meet the energetic demands of an individual. In response to unexpected environmental changes, glucocorticoids concentrations increase rapidly to high 'stress-induced' concentrations that redirect the physiology of an individual into an emergency state to prioritize survival over other less essential activities. Graph adapted from Romero et al. (2009).

The oxidative status of an organism is determined by the concentrations of pro-oxidants and antioxidants present in cells or tissues (Figure 1.3a; reviewed by Costantini 2019). Pro-oxidant compounds, also known as reactive oxygen species, are oxygen radicals and non-radical derivatives of oxygen which are mainly produced by normal metabolic activity of mitochondria (e.g., Monaghan et al. 2009, Costantini 2014). Antioxidants, on the other hand, are defined as substances

that delay, prevent or remove oxidative damage caused by pro-oxidants, and can be divided into two major groups: enzymatic and non-enzymatic antioxidants (Surai 1999, Costantini 2008, Halliwell & Gutteridge 2007, Monaghan et al. 2009). Enzymatic antioxidants present in the cells suppress, remove or transform pro-oxidants into less reactive compounds, and repair damaged molecules like proteins, lipids and DNA. Non-enzymatic antioxidants, which are a mixture of dietary acquired and endogenously produced antioxidants, scavenge pro-oxidants to inhibit chain formation and break chain propagation. Similar to glucocorticoid concentrations, the environment can have a direct effect on the oxidative status of an organism (e.g., Finkel & Holbrook 2000; van de Crommenacker et al. 2011; Marasco et al. 2017). For instance, the concentrations of pro-oxidants and antioxidants can change after fluctuations in ambient temperature that require changes in metabolic rate and thermoregulation (e.g., Costantini et al. 2014). A change in food availability can also impair the antioxidant system by affecting the workload of foraging individuals or the amount of dietary antioxidants acquired (Costantini et al. 2014, but see Beaulieu et al. 2010). If the production of pro-oxidants cannot be counterbalanced by the concentrations of antioxidants, oxidative damage to proteins, lipids and DNA molecules can occur (reviewed by Costantini 2019). Oxidative costs can therefore arise from an increase in pro-oxidants and/or a decrease in total antioxidant concentrations (Figure 1.3b).

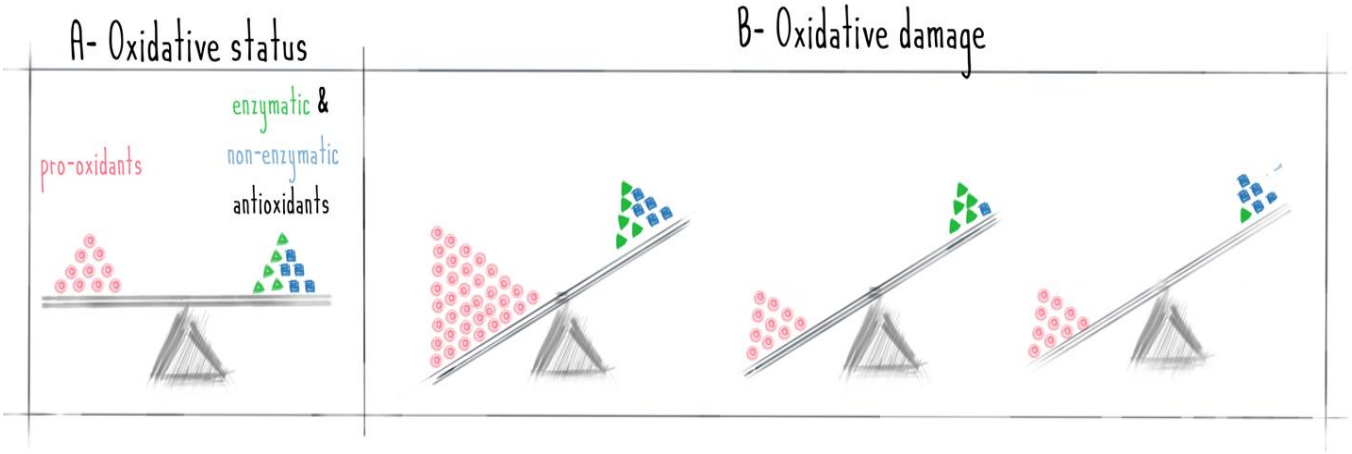

Figure 1.3: The (a) oxidative status of an individual is determined by the concentrations of pro-oxidants (red), enzymatic (green) and non-enzymatic (blue) antioxidants present in cells and tissues. If the production of pro-oxidants cannot be counterbalanced by the concentrations of antioxidants, (b) oxidative damage to biomolecules can occur.

1.4.1. Glucocorticoids and oxidative status can relate to fitness

The concentrations of glucocorticoids and the oxidative status of an organism can thus affect its reproductive outcome and/or survival (Sapolsky et al. 2000; McEwen & Wingfield 2003; Costantini 2008; Monaghan et al. 2009; Metcalfe & Alonso-Alvarez 2010; Romero & Wingfield 2016; Costantini 2018; Vágási et al. 2019). However, over the past years, studies have consistently highlighted the lack of uniform relationships between glucocorticoids, oxidative status and fitness traits in vertebrates (Breuner et al. 2008; Bonier et al. 2009; Metcalfe & Alonso-Alvarez 2010; Crespi et al. 2013; Metcalfe & Monaghan 2013; Speakman & Garrat 2014; Speakman et al. 2015; Blount et al. 2016; Romero & Wingfield 2016; Costantini 2018; Schoenle et al. 2019), suggesting that these relationships are not as direct as previously hypothesized.

Advances in our understanding of the relationship between glucocorticoids, oxidative status and fitness are critically important for evaluating how animals cope with challenges and what constitutes a successful physiological response to a challenge.

1.4.2. Do glucocorticoids and oxidative status relate to fitness in a context-dependent way?

Environmental conditions can directly affect both the physiological condition of the parents and fitness. The inconsistent associations between glucocorticoids, oxidative status and fitness may therefore stem at least partly from a common environmental factor simultaneously affecting both physiological traits and fitness (e.g., Dantzer et al. 2016, Schoenle et al. 2018). For example, male great tits (*Parus major*) had low stress-induced corticosterone concentrations and high fledgling success in the year when food abundance was higher (Ouyang et al. 2013), or in marine iguanas (*Amblyrhynchus cristatus*), males with an improved efficacy of negative feedback on the HPA axis have higher survival but only in El Niño years (Romero & Wikelski 2010). Only recently studies have directly addressed how physiological parameters and reproductive success are linked under varying natural conditions, supporting the idea that the relationships between glucocorticoids, oxidative status and fitness are context-dependent (Simons et al. 2014, Ouyang et al. 2015, Henderson et al. 2017, Vitousek et al. 2018a). Studies measuring the ecological context together with physiological and fitness traits might be key to disentangle if physiological traits indeed relate to fitness or if this relationship is underlined by a common environmental factor.

1.4.3. Do glucocorticoids and oxidative status act as part of an integrative system?

Glucocorticoids and oxidative markers (i.e., any biomolecule that is part of the redox system) can interact with each other within an organism (Esposito et al. 1995; Lin et al. 2004a & Lin et al. 2004b; Costantini et al. 2008; Haussmann & Marchetto 2010; Costantini et al. 2011; Haussmann et al. 2012; Haussmann & Heindinger 2015; Vágási et al. 2018; Majer et al. 2019). Glucocorticoids can increase the metabolic generation of free radicals and decrease both antioxidant defense concentrations and oxidative damage repair systems (e.g., Flint et al. 2007; You et al. 2009; Haussmann and Marchetto 2010; Flaherty et al. 2017). In turn, the intracellular oxidative state can impair the function of the HPA axis by inhibiting the expression of genes encoding glucocorticoids receptors, decreasing the DNA binding activity of the glucocorticoid receptor, and diminishing the negative feedback regulation (e.g., Esposito et al. 1995; Allen & Tresini 2000; Asaba et al. 2004). If glucocorticoids and oxidative markers interact with each other the combined action of both physiological traits, and not each trait separately, might help an organism respond to environmental changes. Yet, whether glucocorticoids and oxidative markers can jointly mediate the relationship between environmental changes, phenotype and fitness is still unclear in wild populations of vertebrates (e.g., Ouyang et al. 2016; Fowler et al. 2018; Casagrande & Hau 2018; Guindre-Parker & Rubenstein 2018). Furthermore, the only study looking at the correlation between both physiological systems in males and females suggests that the interaction is sex-specific (Ouyang et al. 2016).

1.5. How does the way mothers cope with environmental changes affects its reproductive investment and their offspring phenotype?

In many species, the mother is the one that provides the first environment an individual encounters in its life, even before it is born. This prenatal environment depends, to a large extent, on the resources mothers pass on to their offspring, such as hormones, nutrients and immune components (e.g., Noble & Cocchi, 1990; Dloniak et al. 2006; Gil 2008; Groothuis & Schwabl 2008; Surai & Speake 2008; Dupoué et al. 2015; Valcu et al. 2019); resources that largely depend on changes in the environment and the way females respond to these changes. For example, the quantity or quality of food available to mothers can directly impact the amount of energy available for investment into reproduction. In turn, maternal nutrition can affect the nutrients available to developing embryos as well as the number or size of offspring produced (e.g., McCormick 1999; Guisande et al. 2000; Surai et al. 2001; Warner et al., 2007; Lovern & Adams 2008; Gil 2008; Groothuis & Schbawl

2008; Parolini et al. 2019). Similarly, the behavioral response of mothers towards environmental stimuli (e.g., interaction with conspecifics or predators) can influence the transmission of hormones to the developing embryos (e.g., Dloniak et al. 2006; Pitk et al. 2012). The way mothers (and fathers) cope with environmental fluctuations can also influence the environment of the offspring after the egg was laid (e.g., parents can influence the temperature and/or moisture conditions the eggs experience during incubation; Coe et al. 2015) and/or once the offspring is born (e.g., parental feeding behavior, protection from predators; Hunt & Simmons 2000). Hence, because the environment provided by mothers can have a strong and long-lasting influence on the behavior, morphology and physiology of the offspring (reviewed by Moore et al., 2019; Yin et al., 2019), maternal investment has important consequences for both mother and offspring fitness (Mousseau & Fox 1998).

1.6. Birds as models

Birds are excellent models to study the relationship between physiological traits, phenotypic variation and fitness outcomes in response to natural environmental changes. Bird studies have been at the forefront of the development of a mechanistic understanding of life-history diversification in natural contexts. For example, information on the relationship between glucocorticoid concentrations and natural environments is available for more than 115 bird species (followed by reptiles: ~45 species, amphibians: ~25 species, and mammals: ~ 10 species; Vitousek et al. 2019) according to a public database of steroid hormones in vertebrates (HormoneBase; Vitousek et al. 2018b). Compared to mammals, birds also have an exceptional resistance to oxidative stress despite their high metabolic rate (i.e., double than mammals; Costantini 2008; Skrip & McWilliams 2016); thus, making them good systems to study coping mechanism for oxidative stress. Furthermore, bird survival and reproductive success can be relatively easy to study in the field, which allow us to address evolutionary questions under natural scenarios.

Birds are also excellent systems to study the effects that parental responses to the environment have in the offspring phenotype. In egg laying species like birds, embryos develop in the egg, outside the mother's body, within a contained system. Mothers influence the immediate environment in which their offspring will develop mainly before laying, via the deposition of compounds into the egg (e.g., Gil 2008; Groothuis & Schwabl 2008; Surai & Speake 2008). Differences in yolk deposition have been documented across many bird species, at different phenotypic levels (e.g.,

among- and within-populations), and for multiple yolk components (e.g., hormones, nutrients and immune components; Groothuis & Schawabl 2008; Gil 2008; Surai & Speake 2008; Giraudeau & Ducatez 2016). Information on the potential mechanisms of maternal transfer into the eggs is also available (reviewed by Surai & Speake 2008; Groothuis & Schawabl 2008; Gil 2008). After egg laying, parents can influence the environment of the offspring by altering the temperature at the nest (e.g., through incubation; DuRant et al. 2013), by acoustically signaling current climatic conditions (e.g., Mariette & Buchanan 2016), and by the food items they feed their chicks on (e.g., Twinning et al. 2019), among others. Hence, the use of birds as model systems facilitate the measurement of both the direct (e.g., weather conditions) and indirect (e.g., nutrients transferred by the mother into the egg) environment of the developing embryo and makes it possible to separate pre-and postnatal effects on the offspring.

1.6.1. Yolk components

Hormones have pleiotropic effects that can affect a range of phenotypic traits in the offspring. In birds, yolk steroid hormones such as glucocorticoids and androgens (e.g., androstenedione, 5α-dihydrotestosterone, and testosterone) can promote growth, competitive ability and survival (Groothuis et al. 2005; Gil 2008). They can also increase chick susceptibility to oxidative stress by increasing the production of reactive oxygen species or by impairing antioxidant defenses (Haussmann et al. 2012; Treidel et al. 2013). Maternally derived yolk antioxidants, like carotenoids or vitamin E, can influence growth and limit the negative consequences of increased oxidative stress because they can scavenge the reactive oxygen species produced during growth (Surai 2000; Surai 2002; Parolini et al. 2017; Watson et al. 2018). The fatty acids in the yolk supply the avian embryo with almost all of the energy required for development and also provide components for the formation of cell membranes, heart functioning and brain development (Noble & Cocchi 1990, Surai & Speake 2000, Hulbert & Abbott 2011). But they can also lead to an increase in oxidative stress through an increase of reactive oxygen species generated as a by-product of the embryonic metabolism (Pamplona et al. 2002; Larsson et al. 2004; Hulbert & Abbott 2011; Yigit et al. 2014). Yolk steroid hormones, antioxidants and fatty acids therefore influence the same offspring phenotypic traits, such as growth or oxidative status. This opens up the possibility that yolk components interact with each other, with several components jointly determining offspring phenotypes. Recently, a study done in 112 bird species, reported a positive correlation between yolk testosterone and vitamin E concentrations in eggs (Giraudeau & Ducatez 2016); thus,

supporting the idea that egg components are co-adjusted. However, the potential interactive effects of maternally transmitted compounds on offspring development and phenotype remains poorly understood.

1.6.2. Yolk deposition

Egg components are both endogenously produced and externally acquired by the mother. Mothers can synthesize steroid hormones, saturated and monounsaturated fatty acids, while they need to obtain antioxidants and essential fatty acids from the diet (Surai 2000; Raclot 2003; Young & Badyaev et al. 2004; Badyaev et al. 2006a; Badyaev et al. 2006b; Badyaev et al. 2008; Groothuis & Schawabl 2008; Gil 2008; Price et al. 2008; Hulbert & Abbott 2011).

Egg components are accumulated into the egg via different mechanisms. The amount of androgens deposited into the yolk are thought to be independent from the female's own hormonal state, and determined by the production of these hormones in the follicle walls that surround each oocyte (Young & Badyaev et al. 2004; Badyaev et al. 2006a; Badyaev et al. 2006b; Badyaev et al. 2008; Groothuis & Schawabl 2008; Gil 2008). The concentrations of yolk glucocorticoids, antioxidants and fatty acids, which have to reach the ovum via the circulation because they are distantly produced or stored, tend to be positively correlated with the concentrations of these components in the mother's plasma (Noble and Cocchi 1990; Lin et al. 1991; Surai 1999; Raclot 2003; Surai et al. 2008; Groothuis & Schawabl 2008; Price et al. 2008; Surai & Speake 2008).

The concentration of yolk components is strongly affected by the changes in the environment, irrespective of whether they are produced by their own mother or obtained through the diet. For example, mate attractiveness, social density, and predation pressure can influence the concentration of steroid hormones present in the eggs (reviewed by Groothuis et al. 2005, Gil 2008), while changes in food availability can influence the concentrations of antioxidants and fatty acids present in the eggs (Lin et al. 1991, Hulbert and Abbott 2011, Twining et al. 2016). The way females respond to environmental stimuli can therefore have a strong influence in the overall composition of eggs, thus affecting the environment under which the offspring will develop.

In most bird species, females lay more than one egg during each breeding event and the concentrations of yolk components vary along the laying sequence. Hence, offspring from the same clutch can develop in different egg environments. Phenotypic variation in yolk deposition along

the laying sequence has been interpreted as a consequence of their availability in the female's diet during the egg laying process (Royle et al. 1999, 2003: Møller et al. 2000; Blount et al. 2002; Hõrak et al. 2002; Rubolini et al. 2011; Török et al. 2007; Surai & Speake 2008) or in light of sibling competition (e.g., in bird species that show asynchronous hatching an average increase in androgens concentrations over the laying sequence could counterbalance a potential disadvantage starting of chicks from late-hatching position compared to its early-hatching siblings; Groothuis & Schwabl 2008; Gil 2008). However, opposing patterns of deposition have been reported for the same species (e.g., great tits, *Parus major*; Tschirren et al. 2004; Groothuis et al. 2008; Lessells et al. 2016). This lack of agreement among studies done on the same species blurs our understanding of the processes that shape female deposition, and on the consequences that maternal components have on offspring phenotype and fitness.

1.7. Thesis outline

Environmental changes can be challenging for an organism because they can destabilize its homeostatic processes. Physiological traits are important mechanisms to cope with fluctuating environments (Zera & Harshman 2001, Ricklefs & Wikelski 2002). Physiological signals serve to integrate information from the environment and the genome, transmitting that information within the organism to mediate a phenotypic response. These responses can have direct fitness consequences for the organisms and can also lead to transgenerational effects (i.e., by affecting the phenotype and survival of the offspring), potentially shaping evolutionary processes.

The general aim of my thesis was to determine the fitness consequences of physiological responses to environmental variation. For this, I studied how the physiological response of parents to environmental conditions relates to patterns of reproductive investment, reproductive success and offspring fitness in a wild population of great tits (*Parus major*).

Glucocorticoids and oxidative status markers respond to environmental signals and help maintain allostasis in an organism. Because of this, glucocorticoid concentrations and markers of oxidative status have been proposed as mediators of the link among environmental changes, phenotypic variation and fitness. However, non-significant, positive and negative relationships between glucocorticoids, oxidative status and fitness have been reported. Because the environment has a dual role, it creates both phenotypic variation and selects among that variation, it has become apparent that we need to quantify the ecological conditions that individuals experience as a way to

understand whether glucocorticoids and oxidative status indeed relate to fitness. From the few studies to date that have done so (e.g., Ouyang et al. 2015; Henderson et al 2017; Vitousek et al. 2018a), it seems that the link between the physiological condition of an individual and its fitness outcome is most apparent under challenging environmental conditions; potentially explaining the mixed results reported to date. For example, in a three-year study on blue tits (*Cyanistes caeruleus*) baseline corticosterone concentrations were positively associated with fledgling success only in a 'bad' year with high rainfall and low temperatures (Henderson et al. 2017). Alternatively, this lack of agreement across and within species could be explained by glucocorticoids and oxidative markers interacting with each other and acting as an integrative system responsible for helping an individual cope with environmental changes. Therefore, in chapter 2, I tested the hypothesis that six physiological traits (i.e., glucocorticoids: baseline and stress-induced corticosterone concentrations, oxidative status: pro-oxidants, dietary and enzymatic antioxidants, and body condition) covary, and that they predict fitness proxies primarily under challenging environmental conditions. As a general conclusion of this chapter, I found that physiological traits do not covary but they predict, in a sex-specific way, reproductive success only when environmental conditions are challenging.

The way in which mothers cope with environmental changes can affect patterns of reproductive investment by altering the resources mothers pass on to their offspring, such as egg hormones, nutrients and immune components. In species that produce more than one egg per clutch, females vary in the concentrations of components they deposit along the laying sequence. Hence, depending on the egg in which they develop, offspring from the same clutch can be exposed to different environments during embryonic development. In birds, the patterns of deposition along the laying sequence vary across and within species, but the processes that shape female yolk deposition are not yet fully understood. In chapter 3 (Figure 1.4b) I employed a statistical approach based on repeated measures that enabled me to study variation in female deposition at different hierarchical level, such as at the population versus the among-female (within-population) levels. My approach hinged on the notion that patterns of female deposition at a population level might be driven by processes at the female level, and since natural selection operates at the individual level, accounting for variation of individual females is of key importance. In particular, I employed mixed effect models to analyze at both levels the variation in deposition along the laying sequence of four steroid hormones, three antioxidants, and four groups of fatty acids in the egg yolks of wild great tits. I

found that the patterns of deposition at the among-female level differ from the pattern observed at the population level, indicating that the developmental environment is different for offspring growing in first versus consecutive eggs. Females were remarkably consistent and plastic in the deposition of the majority of yolk components along the laying sequence, and for some components these two traits covaried. My results show that variation in yolk deposition at a population level is underpinned by different individual patterns, and that females might be constrained in how much of a component they deposit on average into the eggs and how plastic such deposition can be along the laying sequence.

The prenatal environment provided by the mothers to the developing embryos can affect several phenotypic traits in the offspring (e.g., morphology, behavior, and physiology) and have fitness consequences. Remarkably, different yolk components can influence the same offspring traits and potentially interact with each other. For example, in Japanese quail (*Coturnix japonica*) hatchling mass was reduced and oxidative stress increased in chicks hatching from eggs injected with either testosterone or carotenoids (Giraudeau et al., 2016), but when both components were administered together neither hatchling mass nor oxidative stress were affected. However, the potential interactive effects of maternally transmitted compounds on offspring development and phenotype remains poorly understood. In chapter 4 (Figure 1.4c) I built on the knowledge obtained from my work in the previous chapter to investigate the consequences that 31 yolk components had on fitness proxies and offspring phenotypic traits, in particular nestling growth and oxidative status. Overall, my study provides the first evidence for a relationship between yolk fatty acids and offspring fitness proxies in a wild population; yolk components that are strongly influenced by the quality and amount of food ingested by mothers during egg laying. This chapter also supports the idea that offspring phenotypes are the consequence of intricate interactions among yolk components that females deposit into their eggs.

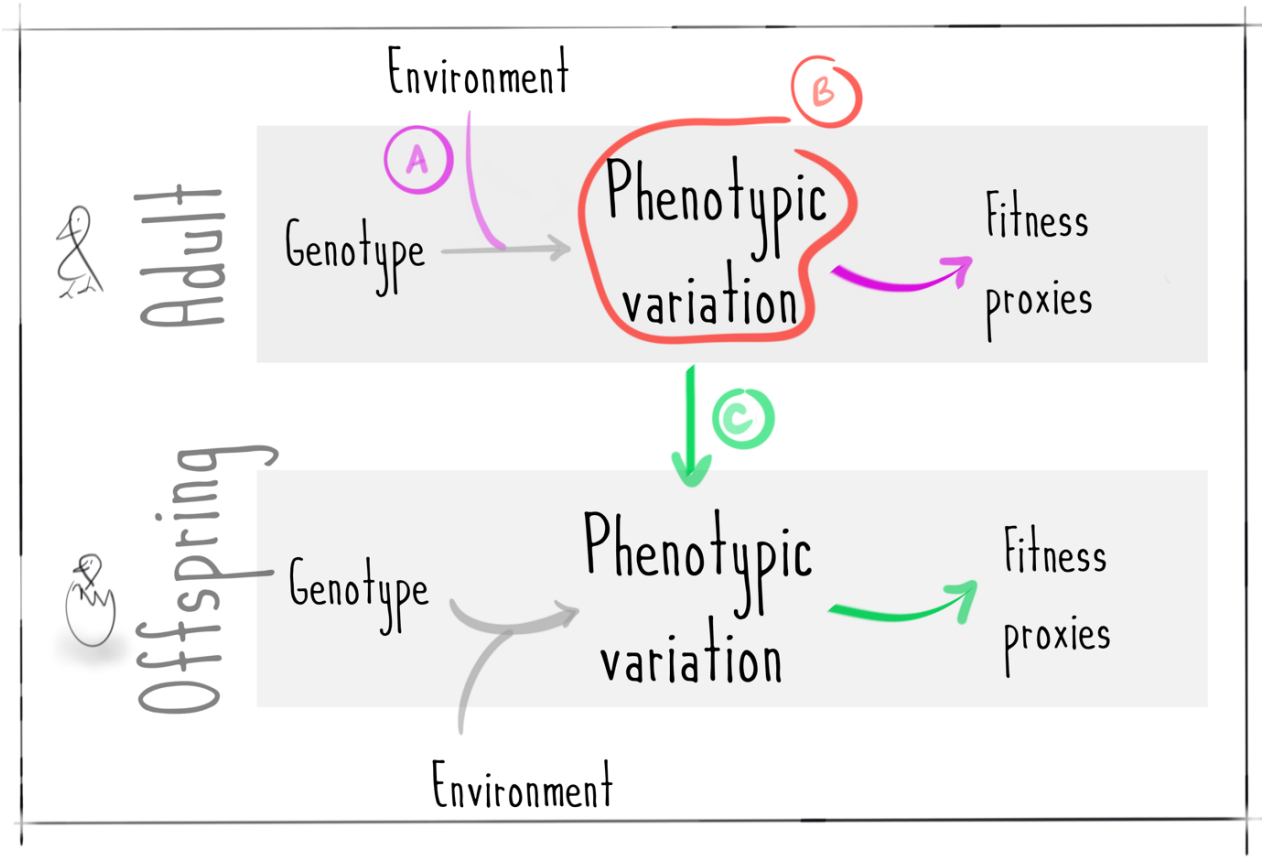

Figure 1.4: For my thesis, I studied the fitness consequences of physiological responses to environmental variation in a wild population of birds. In particular, I first studied (a) the relationship between phenotypic variation in physiological traits and reproductive success in great tit adults over two years with contrasting environmental conditions. Then, (b) I studied female yolk deposition at different hierarchical levels (i.e., population *vs* among-female levels). Lastly, (c) I studied the consequences that maternal effects via yolk deposition had on offspring phenotypic and fitness traits.

1.7.1. Study species

The great tit is a small (ca. 19 gram) passerine bird that is widespread throughout Europe, Asia and North Africa (Figure 1.5; IUCN, 2014). It is a non-migratory bird from the Paridae family, and the most widespread species of its genus. Great tits are cavity nesters that readily breed in nest boxes between March and June (Krebs 1982). This bird is socially monogamous. Shortly after the nest is completed, females start laying one egg per day early in the morning. Females can lay up to two clutches. Great tits eggs weight approximately 1.5 grams and have red-brown speckled shells. Final clutches consist of 6-12 eggs, which are incubated for 12-15 days (Gosler 1993). Great tits are altricial birds (Kölliker et al. 2000). From hatching till fledging (approximately 20 days) nestlings are fed by their parents. While having a seed-dominated diet in winter, great tits feed primarily on

caterpillars during the breeding season (Royama 1970). Caterpillars are also the main food item of the nestlings. Because caterpillars an important source of proteins and fatty acids (Isaksson and Andersson 2007, Andersson et al. 2015, Isaksson et al. 2015), the amount of this food item provided by the parents to their young has an important impact on nestling growth, fledging weight and survival, among others (Naef-Daenzer et al. 1999). Alternatively, parents can also feed their young with spiders and moths (Naef-Daenzer et al. 2000).

Figure 1.5: My study species was the great tit. (a) Adult great tits are small passerine birds. During the breeding season, (b) females can lay up to 12 eggs that weigh around 1.5 gram. From (c) hatching till (d) fledgling both parents take care of the nestlings.

1.7.2. Study area

For my thesis, all data were collected in a nest box population of great tits established in 2014 in the Dellinger Buchet, Southern Germany (Bavaria; 48˚03' N, 11˚13' E, 620 m above sea level). The study site contains 125 nest boxes distributed over an area of approximately 1.30km^2, placed on tree trunks at a height of 1.5-2.0 m and at intervals of approximately 40 m. This area is characterized by a mosaic of deciduous and coniferous forest, with spruce (*Picea sp*.), beech (*Fagus sp.*), ash (*Fraxinus excelsior*), and European larch (*Larix decidua*) as the main tree species (28.31, 28.08, 14.89 & 12.97%, respectively; personal communication with the forester).

Chapter 2

Parental physiological traits do not covary but predict reproductive success under challenging environmental conditions in a wild passerine

Lucia Mentesana, Stefania Casagrande & Michaela Hau

2.1. Abstract

Physiological traits such as glucocorticoid hormones, parameters of cellular oxidative status and body condition have been used to predict individual fitness proxies like reproductive success. However, across studies these traits relate differently to fitness, both in whether a relationship is detectable as well as in its direction. This divergence may be explained by variation in the physiological state of study individuals in addition to the environmental fluctuations that are present in many studies. Here we tested the hypothesis that physiological traits covary, and that they predict fitness proxies primarily under challenging environmental conditions. In free-living adult great tits (*Parus major*), we first determined relationships among circulating baseline and stress-induced glucocorticoid concentrations, oxidative status parameters (i.e., concentrations of pro-oxidants, dietary and enzymatic antioxidants in the blood) and body condition during the parental phase in two years that significantly differed in environmental conditions. We then studied the relationship of these physiological traits with reproductive success (fledgling number and mass). Apart from the two glucocorticoid traits, there were no associations among the physiological measures. Confirming our hypothesis, the relationship between physiological traits and reproductive success was present only in the year with the more challenging weather (lower ambient temperature, higher rainfall), in a sex-specific way. Females in a better oxidative condition produced more and heavier fledglings, while males with high body condition fledged more offspring. In males, high baseline and low stress-induced glucocorticoid concentrations were also related to high fledgling mass. Overall, our study shows that physiological traits do not covary in free-living great tit parents, but supports the view that they predict, in a sex-specific way, reproductive success when environmental conditions are challenging.

2.2. Introduction

How can an individual maximize its fitness in unpredictable environments? Understanding the mechanisms underlying life history variation among individuals of a population is a major goal in evolutionary biology. Physiological traits are important mechanisms to cope with fluctuating environments (Zera & Harshman 2001, Ricklefs & Wikelski 2002). Physiological signals serve to integrate information from the environment and the genome, transmitting that information within the organism to mediate a phenotypic response. However, identifying general patterns in how physiological parameters shape life history traits has proven difficult. Substantial variation exists across studies in the type of physiological trait and the direction of its relationships with life history traits (e.g., Breuner et al. 2008, Bonier et al. 2009, Metcalfe & Alonso-Alvarez 2010, Crespi et al. 2013, Metcalfe & Monaghan 2013, Speakman & Garrat 2014, Speakman et al. 2015, Blount et al. 2016, Romero & Wingfield 2016, Costantini 2018, Schoenle et al. 2019). In wild populations, these contradictory results may be a consequence of either extrinsic factors (i.e., the overall quality or current conditions of the environment) or of individuals differing in their intrinsic (physiological) state, as well as in their individual optima, in response to environmental changes.

Glucocorticoid hormones (GCs), cellular parameters of oxidative status, and body condition have been studied as prominent candidates for mediating life history variation (Schantz et al. 1999, Sapolsky et al. 2000, McEwen & Wingfield 2003, Costantini 2008, Monaghan et al. 2009, Romero & Wingfield 2016; Costantini 2018, Vágási et al. 2019). GCs play a fundamental role in maintaining organismal homeostasis through change (‘allostasis”, McEwen & Wingfield 2003, Romero et al. 2009, Angelier & Wingfield 2013, Romero & Wingfield 2016). At baseline levels, GCs are important for coping with daily life processes and predictable life history stages (Landys et al. 2006). They are also responsible for supporting energetic needs by mobilizing energy reserves or changing behavioral performance. In response to unpredictable perturbations in external or internal conditions, the secretion of GCs increases above baseline levels. This stress-induced GC response can activate alternative physiological and behavioral strategies that allow an organism to survive an acute challenge, and can include the inhibition of non-vital processes like reproduction (McEwen & Wingfield 2003, Wingfield & Sapolsky 2003, Romero & Wingfield 2016). The oxidative status of cells is determined by the concentrations of reactive oxygen species produced as a by-product of normal metabolic processes and the concentration of antioxidants, which are defined as substances that delay, prevent or remove oxidative damages caused by pro-oxidants

(reviewed by Costantini 2019). A disturbance in the balance between the production of reactive oxygen species and antioxidants in favor of pro-oxidant concentrations can damage important molecules like proteins, lipids and DNA (reviewed by Costantini 2019). Lastly, body condition provides information about an individual's energy reserves. It is an integrated trait that results from the outcome of the trade-off between ecological (e.g., food availability or predictability, predation, social status; Gosler 1996, Wikelski & Thom 2000, Brown & Sherry, 2006, Walters et al. 2017, Lázaro et al. 2017) and internal factors (e.g., fat reserves, health status, metabolic rate; Pond 1978, Daan et al. 1990, Gosler 1996, Witter & Cuthill 1997, Schulte-Hostedde et al. 2001, Sánchez et al. 2018).

GCs, oxidative status markers and body condition can vary substantially with environmental conditions. For instance, when ambient temperatures are low baseline GC concentrations can rise (e.g., Romero et al. 2000, Ouyang et al. 2012, Jimeno et al. 2017, 2018b, 2018c) and body mass decrease (e.g., Romero et al. 2000, Jimeno et al. 2017, but see Witter & Cuthill 1997) because of the increased energy demands of thermoregulation and foraging. Increased metabolic rate during low ambient temperatures in turn can lead to higher concentrations of pro-oxidants, thereby affecting the concentrations of exogenously acquired (i.e., dietary) and endogenously produced (i.e., enzymatic) antioxidants (e.g., Christe et al. 2012, Costantini 2014). The duration (and severity) of environmental conditions can influence the magnitude of these changes (Romero et al. 2000; Lushchak 2011; Krause et al. 2018; Schoenle et al. 2018), suggesting that the physiology of an individual is adjusted differently to short- *vs.* long-term environmental conditions. However, only recently studies have directly addressed how physiological parameters and reproductive success are linked under varying natural conditions (e.g., one-year study: Ouyang et al. 2015; multiple years: Ouyang et al. 2012, Henderson et al. 2017, Vitousek et al. 2018a). These studies suggest that the link between physiological and life history traits is primarily present when environmental conditions are challenging. For example, in a three-year study on blue tits (*Cyanistes caeruleus*) baseline corticosterone levels during the parental phase were positively associated with fledgling success only in a year with high rainfall and low temperatures (Henderson et al. 2017). On the other hand, physiological traits can also vary in response to internal changes occurring in the organism. GCs, oxidative state, and body condition parameters can interact with each other and therefore may not exert their actions independently (e.g., Haussmann & Marchetto 2010, Costantini et al. 2011a, Haussmann and Heindinger 2015). For example, GCs can directly affect the oxidative

status of an individual by increasing the metabolic generation of free radicals, while decreasing both concentrations of antioxidant defenses and oxidative damage repair systems (Haussmann & Marchetto 2010). In turn, the intracellular oxidative state can impair the activity of glucocorticoid receptors (Esposito et al. 1995). In addition, interactions between GCs and oxidative markers with body condition have also been reported (e.g., Lendvai et al. 2014, Vágási et al. 2018). Only few studies to date have tested whether GCs, oxidative state and body condition parameters form an integrate physiological phenotype in unmanipulated individuals from natural populations - and with mixed results. While in tree swallows (*Tachycineta bicolor*) a negative relationship between GCs and oxidative status was found in females (but not in males; Ouyang et al. 2016), no covariance between the two traits was present in either female European starlings (*Sturnus vulgaris;* Fowler et al. 2018) or both sexes of cooperatively breeding superb starlings (*Lamprotornis superbus;* Guindre-Parker & Rubenstein 2018). Thus, in wild populations it is still unclear whether different physiological traits act as part of an integrated system and are jointly responsible for maintaining organismal allostasis or, if instead, there is little covariation and each trait has independent effects. Because physiological traits can vary with internal and environmental conditions, gaining a true understanding on how physiological responses relate to life history decisions and fitness in wild populations therefore requires studies that concomitantly measure multiple traits, and along variation in external conditions. Such studies should also be conducted in both males and females because the sexes can differ in their responses to varying internal and external conditions (e.g., Ouyang et al. 2012, Ouyang et al. 2013, Ouyang et al. 2016, Vitousek et al. 2018a).

Here, we studied a free-living population of great tits during two breeding seasons that significantly differed in environmental conditions. First, we tested the prediction that different physiological traits act as part of an integrated system using a multivariate approach (Figure 2.1: a). To do so, we studied the relationships among six physiological traits (GCs: baseline and stress-induced corticosterone levels, oxidative status: pro-oxidants, dietary and enzymatic antioxidants, and body condition). Second, we tested the prediction that the relationship between physiological traits and reproductive output in free-living male and female great tits is most apparent under challenging environmental conditions (e.g., Henderson et al. 2017). For this, we investigated the relationships between parental physiological traits and two fitness proxies (i.e., number and mass of fledglings; Figure 2.1: b), testing whether these relationships differed between study years and sexes. Third,

we analyzed if weather conditions (ambient temperature and rainfall) were directly associated with reproductive success (Figure 2.1: c). Fourth, we asked how weather conditions at two time scales, at capture and in the three days leading up to capture (i.e., short- *vs* long-term conditions), explained variation in the physiological condition of our focal birds (Figure 2.1: d).

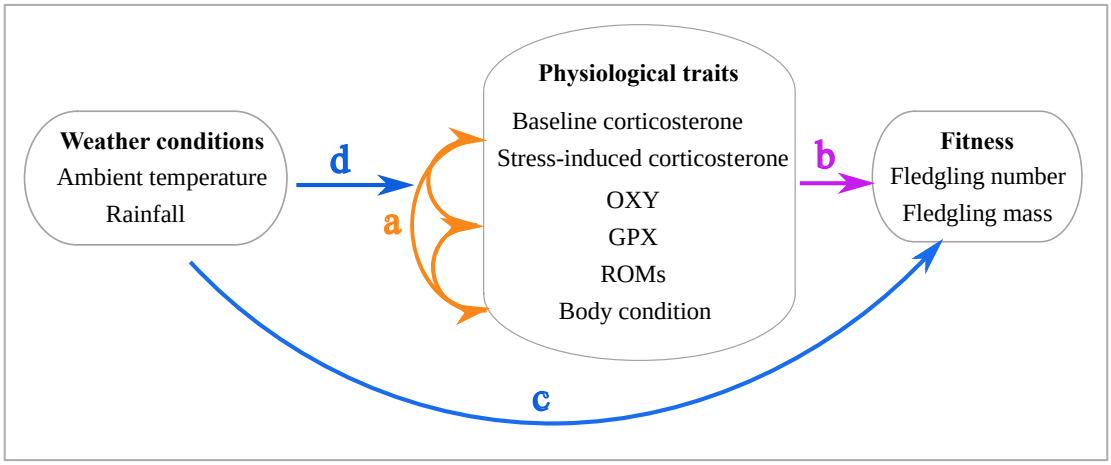

Figure 2.1: Schematical representation of the study goals and analytical steps. We first examined whether (a) the different physiological parameters (GC concentrations, oxidative status, and body condition) were associated with each other, and (b) if these parameters predicted fitness proxies. Third, we studied the relationships between short- and long-term weather conditions with (c) fitness proxies, and (d) the physiological condition of the adults. Oxidative status parameters: OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs, reactive oxygen metabolites in plasma.

2.3. Materials and methods

2.3.1. Ethics statement

All experimental procedures were conducted according to the legal requirements in Germany and were approved by the governmental authorities of Oberbayern, Germany.

2.3.2. Study site, standard protocols and reproductive success

We studied female and male great tits when they cared for their offspring in a nest box population in the Dellinger Buchet, a mosaic of deciduous and coniferous forest patches in Southern Germany (Bavaria; 48˚03’ N, 11˚13’ E, 620 m above sea level), from April to July in 2015 and 2016. Nest-boxes were monitored so that the date of the first egg and the date of hatch (= chick day 1) could

be determined (see Mentesana et al. 2019 for a detailed description of the study site and standard protocol for monitoring nest-boxes). Since most parents only had one brood per year, in the present study only first clutches were considered in the analysis. On day 9 after hatching, adults were captured inside their nest-boxes between 8:00 – 12:00 am with a remote-controlled trap that closed the nest entrance after parents entered to feed their young. A blood sample (max. 80 µl) was taken within 3 minutes of capture (mean ± SD: 2.36 ± 0.51) for later determination of baseline corticosterone concentrations and oxidative status of each individual. Birds were then placed in a cloth bag for a standard capture and restraint protocol (Wingfield et al. 1982) and another blood sample (<40 µl) was taken at 30 minutes post capture to determine stress-induced corticosterone concentrations. After taking the second blood sample, birds were individually marked with a numbered aluminum ring and plastic split rings with a unique color combination. Body mass (to the nearest 0.1 g), tarsus length (to the nearest 0.1 mm) and wing length (to the nearest 0.1 mm) were measured for each captured individual. Scaled body mass index was calculated as an indicator of adult body condition (Peig and Green 2009). This index represents a measure of body mass relative to body size and thus can serve as a proxy for the amount of energy reserves an individual carry. As recommended by Peig & Green (2009), parameters with the highest correlation in our population were used to estimate body condition, which were body mass and wing length ($r = 0.50$; p-value < 0.01). Adult age (first versus experienced breeders) was determined from plumage characteristics or from banding records. However, we excluded this parameter from our final analyses since none of the physiological traits measured varied across age. Adults were then released at the site of capture.

For the purpose of our study, two proxies of reproductive success were used. First, fledging number was determined by monitoring nests every 5 days until fledging date. Second, nestling body condition was estimated on day 15 after hatching by measuring body mass (to the nearest 0.1 g), tarsus length (to the nearest 0.1 mm) and wing length (to the nearest 0.1 mm). Given that the correlation between nestling mass and tarsus or wing length was low, and that mass at fledging is a key predictor of post-fledging survival in great tits (Tinbergen & Boerlijst, 1990), we only considered nestling mass in our analyses.

2.3.3. Corticosterone hormone analysis

Plasma corticosterone concentrations were determined using enzyme immunoassay kits (Lot No: 2015: 12041402C, 2016: 12021512B; Enzo Life Sciences GmbH, Germany) following a double diethyl ether extraction of 10 µl and 7 µl plasma aliquots for baseline and stress-induced levels, respectively (see Baugh et al. 2014 for further details on this protocol). Along with the samples, a blank and two positive controls with each 14 µl of stripped-chicken plasma (at a concentration of 20 ng/ml) were then re-dissolved in 280 µl of assay buffer, reconstituted overnight and taken through the entire assay. The next day, duplicates of 100 µl were added to individual wells. We randomized the position of the samples across 21 plates. The intra-assay coefficients of variation, determined from two positive controls per assay, was (mean ± SD) = 10.57 ± 7.95%, and the inter-assay variation, determined from the first positive control per assay, was (mean ± SD) = 11.55 ± 8.86%.

2.3.4. Oxidative status analyses

The oxidative status of the captured individuals was determined from reactive oxygen species present in plasma, and non-enzymatic and enzymatic antioxidants present in plasma and red blood cells, respectively. Oxidative damage compounds were measured using the d-ROMs test kit (Diacron International SRL, Grosseto, Italy) that quantifies the concentration of reactive oxygen metabolites (i.e., ROMs - the products of free radical reactions with biological macromolecules) following the protocol described by Costantini et al. (2006). Reactive oxygen metabolites are expressed as mM H_2O_2 equivalents. Antioxidant plasma concentrations (i.e., OXY – quantifies the ability of antioxidants to cope with the oxidant action of hypochlorous acid (HOCl)) were measured by the OXY-Adsorbent test (Diacron International SRL, Grosseto, Italy) following the protocol described by Costantini et al. (2006). Concentrations are expressed as mM HOCl neutralized. The activity of glutathione peroxidase (GPX) present in red blood cells was determined using the Ransel assay (Randox Laboratories, Germany) following Costantini et al. (2011b). Measurements are expressed as U/ml of red blood cells.

2.3.5. Assessment of environmental conditions

We monitored ambient temperature and rainfall to assess the weather conditions experienced by great tits during the breeding season. Ambient temperature (°C) was recorded every hour by 12 i-buttons (DS9093A+ Thermochron iButton) placed in different locations in the forest, while

information on total rainfall (mm) was obtained from a meteorological station less than 5 km from our field site (Oberpfaffenhofen; 48° 05' N, 11 ° 16' E, 583 m above sea level). To assess the impact of short- and long-term weather conditions on the physiological variables we used ambient temperature and cumulative (i.e., daily sum) rainfall at the time of capture, and mean ambient temperature and the mean rainfall experienced in the 72hrs prior to blood sampling. Lastly, to determine the impact of environmental conditions on the fitness proxies (i.e., number and mass of the fledglings) we used the mean ambient temperature and rainfall experienced by each brood during the nestling phase (i.e., from day 1 to 20 after hatching).

2.3.6. Statistical analysis

First, to investigate associations among all six physiological parameters (Figure 2.1: a) we ran a Principal Component Analysis (PCA-correlation matrix). PCA is a common data reduction method that can be used to characterize the correlation structure of a set of variables (Paliy and Shankar 2016). We initially ran a PCA for each sex separately, but because the results obtained were similar (results not shown), we present the outcome obtained after pooling the sexes. For each principal component we only considered variables that had an absolute value of squared loadings of higher than 0.3. Principal components one (PC1), two (PC2), and three (PC3) accounted for 59% of the cumulative variance in our data.

The results from the PCA analysis indicated that each group of physiological traits variables was represented in different PCs, suggesting weak associations among GCs, oxidative status and body condition. Also, within each principal component an examination of its loadings did not provide a clear biological interpretation (Table 2.1, Results Section). Thus, to study our second aim, that is whether parental physiological traits explained variation in breeding success (Figure 2.1: b), we decided to run three separate models that each included the variables belonging to one physiological group (i.e., GCs, oxidative stress and body condition). Females and males were analyzed in separate models to avoid over-parametrization. Generalized linear mixed models with a Poisson distribution were used to study fledgling number in relation to physiological traits of the parents. Physiological traits were fitted as covariates, while year (2015 *vs* 2016) was included as a fixed factor. The interactions between each physiological trait and year were included as well. Band number was fitted as random factor. To study the relationship between fledgling mass and the physiological condition of the parents we used linear mixed models. Brood size had a strong effect

on fledgling mass (results not shown). Therefore, to control for this without overfitting the model we used the residuals of a linear regression between fledgling mass and brood size as the dependent variable. Each physiological variable was fitted as a covariate, and year was fitted a fixed factor. The interaction between these two variables was also included in the model, and nest ID was fitted as random factor. It is important to remark that the mean weather conditions experienced by the parents during the breeding season (Supplementary information 9.1, Table 4) were included as covariates in the final models since none of them had an effect on reproductive success.

Third, to assess the direct effect that the overall weather conditions had on fledgling number and mass (Figure 2.1: c) we ran a generalized linear-mixed effect model with a Poisson distribution and a linear-mixed effect model, respectively. For the model analyzing fledgling mass as a fitness proxy we used the residuals of a linear regression of the mass of each fledgling and brood size as the dependent variable. Mean ambient temperature and mean cumulative rainfall during the nestling period were only weakly correlated ($r = 0.19$, $p = 0.15$). Thus, both variables were included in the same model. Date was excluded from the model since it was positively correlated with both environmental variables (mean ambient temperature: $r = 0.65$, $p < 0.00$; mean cumulative rainfall: $r = 0.46$, $p < 0.00$). Year and the interaction of this variable with both environmental variables were also included in the model as explanatory variables. Band number was fitted as random factor in the models using fledgling number as a fitness proxy, while nest ID was used in the models studying the effect of weather conditions on fledgling mass. To study the impact of short- and long-term weather conditions on the physiological condition of the adults (Figure 2.1: d) we ran linear mixed-models fitting each physiological parameter as a response variable. We used ambient temperature and cumulative rainfall at time of capture to study the impact of short-term weather conditions and mean ambient temperature and the mean rainfall experienced during the 72hrs prior to capture to study the effect of long-term weather conditions. Ambient temperature and cumulative rainfall were negatively correlated at both times scales (short-term: $r = -0.30$, $p < 0.00$; long-term: $r = -0.51$, $p < 0.00$). Therefore, models were run twice, once with rainfall and once with temperature as covariates. Date was also fitted as a covariate. Sex (females *vs* males) and year were included as fixed factors, and two-way interactions between each of the covariates and sex and year were also fitted in the model. Band number and nest ID were included as random factors to account for repeated measurements.

All statistical analyses were performed in R statistical freeware R-3.3.3 (R Core Team 2013). The PCA was performed using the "*prcomp*" function from the R-package *"stats"*. Linear and generalized mixed-models were performed using the *"lme4"* and *"arm"* packages in a Bayesian framework with non-informative priors. We assumed a Gaussian error distribution, which was confirmed for all response variables after visual inspection of model residuals. When necessary, response variables were transformed (details on transformations are provided in Supplementary information 9.1). All continuous explanatory variables were mean-centered, and their variance standardized to facilitate comparison of variance components across traits. If no variance was explained, the corresponding random factor was excluded from the final model. We subsequently used the *sim* function to simulate values from the posterior distributions of model parameters. Because interpreting the significances of tests is not trivial in light of the numerous comparisons, we extracted the 95% Bayesian Credible Interval (CrI) around the mean and assessed statistical support by obtaining the posterior distribution of each parameter (Gelman and Hill 2007, Gelman et al. 2012). CrI provide more valuable information than p-values, like for example, the uncertainty around the estimates. We considered an effect size to be "statistically meaningful" when the estimated effect differed from zero with a posterior probability higher than 0.95. The threshold of 5% would be equivalent to significance level in a frequentist framework (for further details on statistical inference see Korner-Nievergelt et al., 2015).

2.4. Results

We sampled 109 adults over two breeding seasons, of which 101 individuals ($N_{females}$ 2015= 8, $N_{females}$ 2016 = 42; N_{males} 2015 = 14, N_{males} 2016 = 37) were captured only once. Fitness proxies (i.e., number and mass of the fledglings) were obtained for 185 chicks (N_{chicks} 2015 = 41, N_{chicks} 2016 = 144) from 40 nests (N_{nests} 2015 = 11, N_{nests} 2016 = 31).

Mean ambient temperatures were higher and mean rainfall were lower during the nestling period of 2015 compared to 2016 (Supplementary information 9.1, Table 1). In 2016 nestlings had lower body mass compared to chicks sampled in 2015. Because differences in weather conditions translated into differences in the condition of the nestlings (Wingfield et al. 2017), hereafter we will consider 2015 as a 'good' and 2016 as a 'bad' year.

2.4.1. Relationship among physiological traits

Using PCA we identified the relationship among all six physiological variables (Table 2.1). The first axis (PC1) explained 22% of the variance and was associated with low levels of both baseline and stress-induced corticosterone levels. The second axis, PC2, explained 20% of the variance and was positively represented by ROM levels and body condition and negatively by OXY levels. Lastly, PC3 explained 17% of the variance and was positively explained by both GPX and ROM levels.

Component	Main variables (direction)	Cumulative variance explained
PC1	Baseline corticosterone (-) Stress-induced corticosterone (-)	22%
PC2	OXY (-) ROMs (+) Body condition (+)	42%
PC3	GPX (+) ROMs (+)	59%

Table 2.1: Main physiological variables contributing to PC1, PC2, and PC3, and cumulative variance explained by each component. OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs, reactive oxygen metabolites in plasma.

2.4.2. Physiological traits and reproductive success

Physiological traits of wild great tits were related to reproductive success only in the 'bad' year (2015), with different patterns observed in the two sexes (Figure 2.2 & 2.3, Supplementary information 9.1, Tables 2 & 3). In females, better oxidative state (low concentrations of reactive oxygen species or high concentrations of antioxidants) was associated with higher reproductive success in 2016. Specifically, females with higher GPX concentrations fledged more chicks (Figure 2.2; Supplementary Table 2), and females with lower ROM levels had heavier chicks (Figure 2.3, Supplementary information 9.1, Table 2) than females with opposite trait values. In contrast, in the 'bad' year (2016), males in better body condition fledged more offspring (Figure 2.2, Supplementary information 9.1, Table 3), and males with high baseline and low stress-induced GC levels produced heavier fledglings (Figure 2.3, Supplementary information 9.1, Table 3).

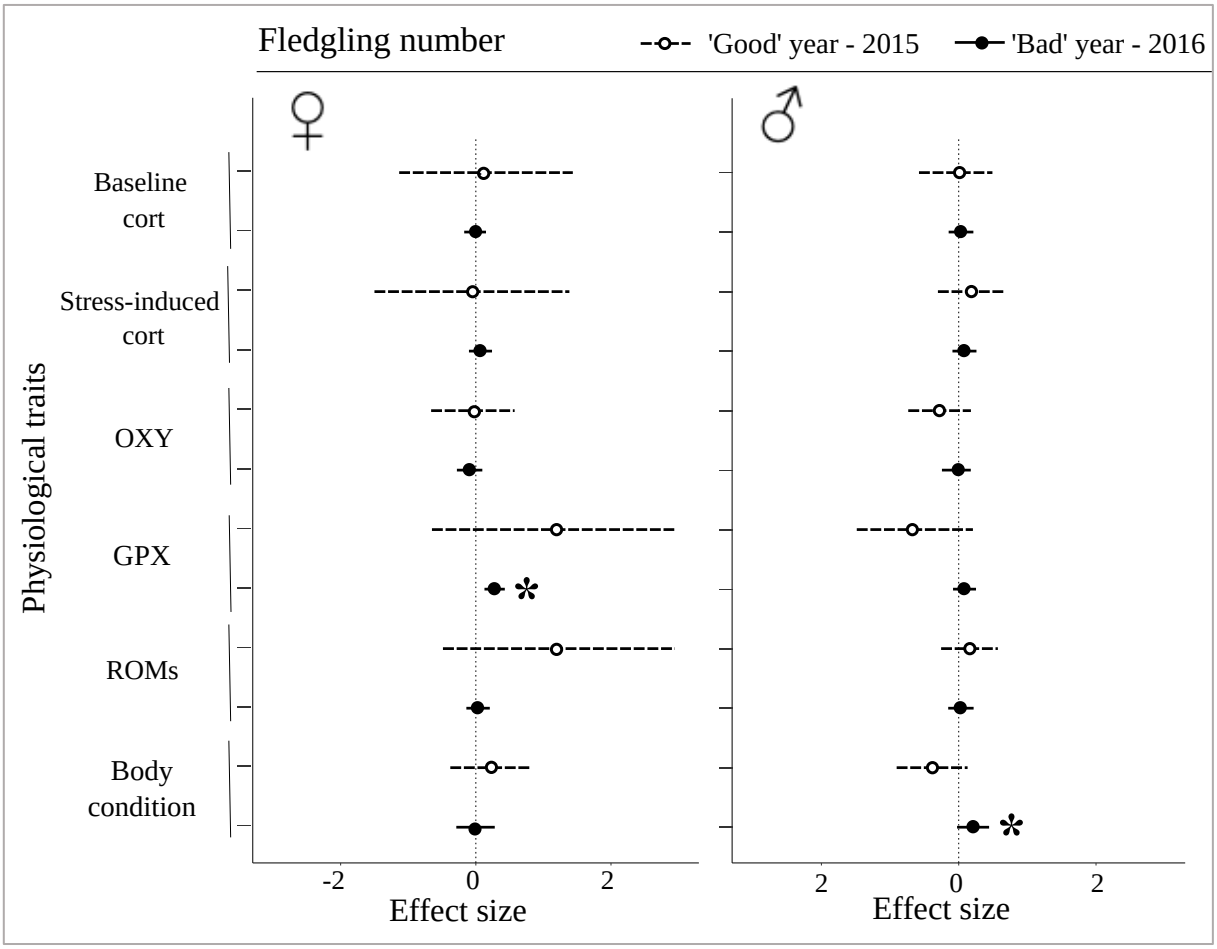

Figure 2.2: Standardized effect size estimates for number of fledglings in relation to corticosterone concentrations, oxidative status, and body condition parameters obtained for males and females from generalized linear mixed models during the breeding seasons with 'good' (i.e., 2015) and 'bad' (i.e. 2016) weather conditions. Open and filled circles represent posterior means for the years 2015 and 2016, and 95% credible intervals (CrI) are indicated in dashed and solid lines for each year. Oxidative status parameters: OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs, reactive oxygen metabolites in plasma.

* Statistically meaningful support (i.e., if the mean difference between compared estimates is higher than 0.95) for an effect of a physiological parameter on the number of fledglings.

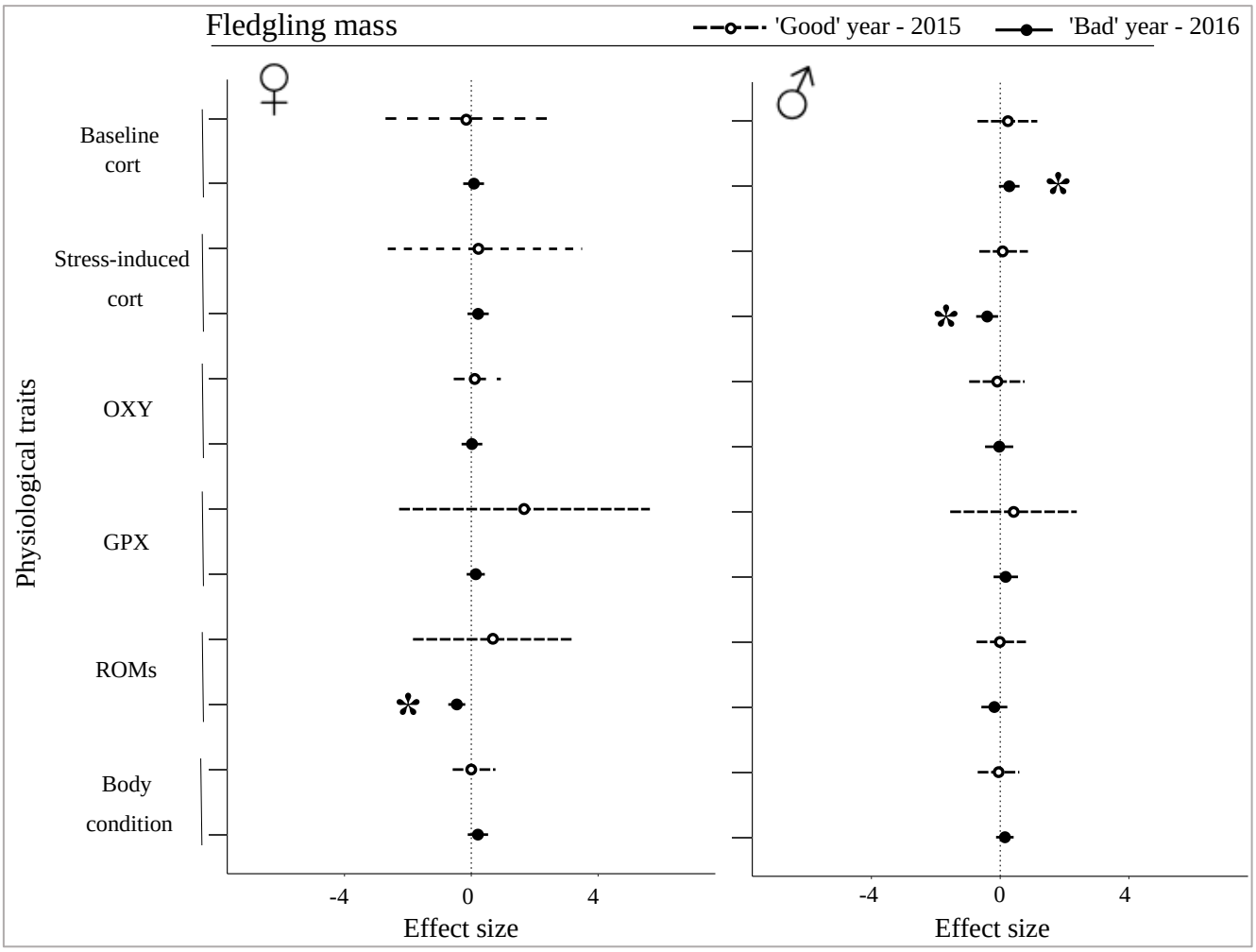

Figure 2.3: Standardized effect size estimates for the mass of fledglings in relation to corticosterone concentrations, oxidative status, and body condition parameters obtained for males and females from generalized linear mixed models during the breeding seasons with 'good' (i.e., 2015) and 'bad' (i.e. 2016) weather conditions. Open and filled circles represent posterior means for the years 2015 and 2016, and 95% credible intervals (CrI) are indicated in dashed and solid lines for each year respectively. Oxidative status parameters: OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs, reactive oxygen metabolites in plasma.

* Statistically meaningful support (i.e., if the mean difference between compared estimates is higher than 0.95) for an effect of a physiological parameter on the mass of fledglings.

2.4.3. Environmental conditions, reproductive success and physiological traits

Fitness proxies were not directly associated with weather conditions (i.e., mean ambient temperature or mean cumulative rainfall) experienced by the parents during the chick rearing period (Supplementary information 9.1, Table 4). However, both short- and long-term weather conditions were linked to the physiology of adult great tits, with differences between years and the sexes. In females, weather was related to a better oxidative state in the 'good' year, with mild long-term temperatures being associated with high GPX concentrations and high rainfall at capture with low ROM concentrations (Figure 2.4, Supplementary information 9.1, Tables 5 & 6). Male physiology appeared to be more responsive to weather than that of females, because in addition to measures of oxidative state variation in corticosterone traits and in body condition was explained by climatic conditions (Figure 2.4, Supplementary information 9.1, Tables 5 & 6). In the 'bad' year of 2016, mild temperatures at capture and over the long-term were related to lower stress-induced GC levels in males and mild long-term temperatures also predicted higher GPX concentrations. Higher long-term rainfall was linked to lower male ROM concentrations. Like in females, in the 'good' year weather conditions were mainly related to measures of oxidative status on males, although patterns were less clear. Similarly to females, in males high rainfall at capture in the 'good' year predicted low ROM concentrations while long-term mild temperatures were linked to high ROM concentrations. In males, higher long-term rainfall was associated with lower GPX levels and worse body condition.

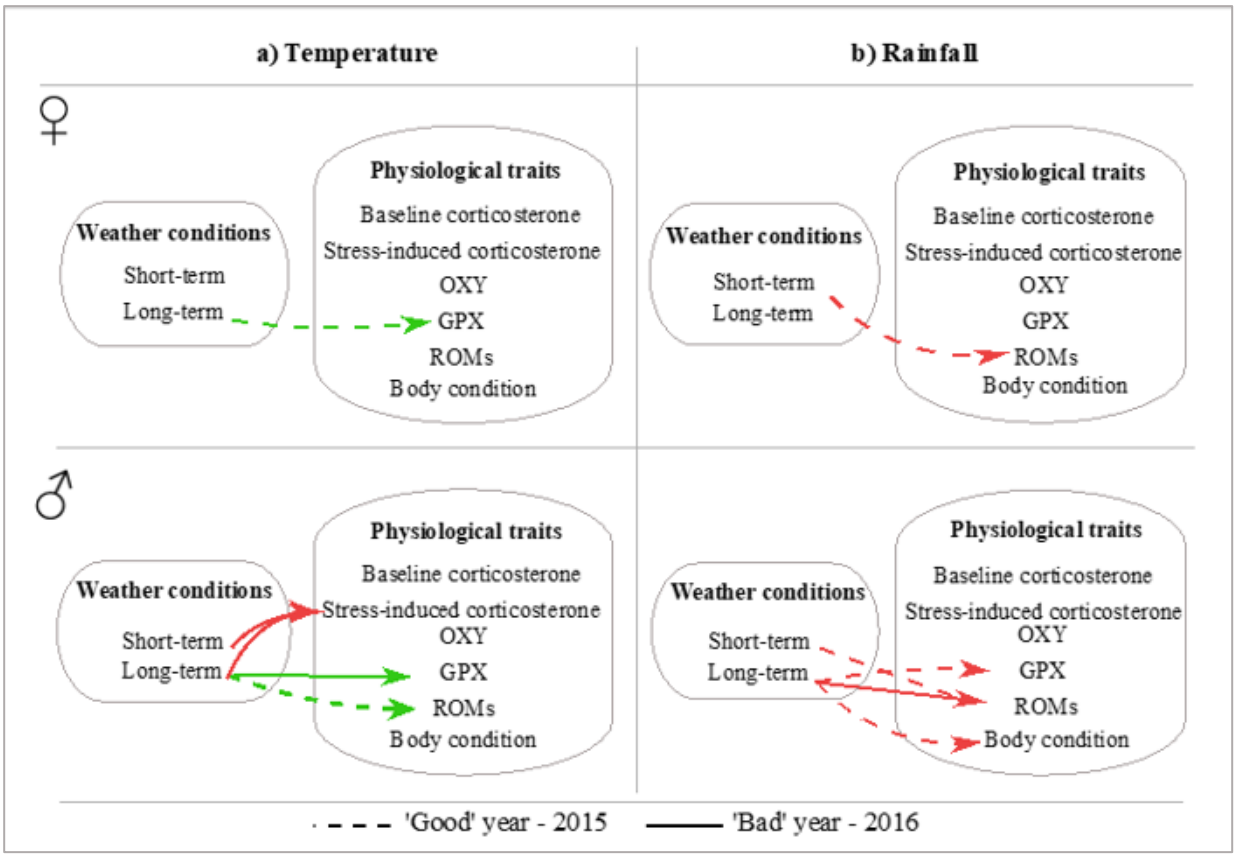

Figure 2.4: Schematical depiction of the relationships between short- and long-term a) temperature and b) cumulated rainfall and physiological conditions in female (top panel) and male (bottom panel) great tits. Red lines indicate positive, and green lines negative relationships. Dashed lines indicate an association in the 'good' (2015), and solid lines in the 'bad' (2016) year. Oxidative status parameters: OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs, reactive oxygen metabolites in plasma.

2.5. Discussion

We found no evidence for the existence of an integrated physiological phenotype in adult great tits during the parental phase; the six physiological traits that we measured showed little covariation. In line with our prediction, physiological traits of parents predicted their reproductive success only in the year with unfavorable environmental conditions. These physiology-fitness relationships were sex-dependent, with high reproductive success being associated with a better oxidative state in females, and with high body condition, high baseline and low stress-induced corticosterone concentrations in males. Climatic conditions did not directly predict reproductive success but were

related to measures of oxidative state in females, and to oxidative state, body condition and corticosterone concentrations in males.

We found little evidence for an association among GCs, oxidative stress and body condition - except for a positive correlation between baseline and stress-induced corticosterone concentrations (Supplementary information 9.2, Figure 1), which is often reported in the avian literature (Ouyang et al. 2013, Grace and Andersson 2014, but see Jenkins et al. 2014). It has been hypothesized that different physiological components work interactively as an integrated system to maintain organismal stability (Ketterson et al. 2009, Cohen et al. 2012, Baugh et al. 2014). A link among physiological variables was reported in experimental studies, where the manipulation of baseline and stress-induced corticosterone concentrations caused increases in oxidative stress levels and/or decreases in body condition in several bird species (e.g., broiler chickens, *Gallus gallus domesticus*, Lin et al. 2004a & Lin et al. 2004b; kestrels, *Falco tinnunculus,* Costantini et al. 2008; house sparrows, *Passer domesticus,* Lendvai et al. 2014, Vágási et al. 2018; but see Vitousek et al. 2018b; Japanese quail, *Coturnix japonica*, Majer et al. 2019). However, the few studies performed under natural, non-manipulated conditions did not universally support these findings (Ouyang et al. 2016, Guindre-Parker and Rubenstein 2018, Fowler et al. 2018). Only one study reported a statistically meaningful relationship between GCs, oxidative state and body condition (Ouyang et al. 2016), and this relationship was sex-specific: female tree swallows with high baseline corticosterone concentrations had low oxidative stress. A relationship between these two parameters and body condition was not detected in either sex. Two non-mutually exclusive explanations for the disagreement between experimental and non-experimental studies are conceivable. First, an experimental manipulation of physiological traits usually creates more extreme phenotypes and thus larger variation in some traits than in natural settings ("phenotypic engineering", Ketterson et al. 1996, Feder et al. 2000). While this can be useful to detect patterns, it might also cause an imbalance in other traits, leading to 'artificial' physiological interactions. Second, in studies on wild populations under natural (i.e., non-manipulated) conditions it might be more challenging to identify associations among physiological traits because these often change substantially, and to a different extent, with natural variation in environmental conditions (see discussion below; e.g., Buehler et al. 2012, Cohen et al. 2012). This physiological flexibility, which allows individuals to cope with environmental fluctuations, might be limiting our capacity to uncover potential

associations among physiological traits with the current methodological approaches used (i.e., by sampling individuals at a single point in time, Fowler et al. 2018; but see Buehler et al. 2012).

Only during the 'bad' year of 2016, with low ambient temperatures and higher rainfall, did physiological traits of the parents predict their reproductive outcome (Figures 2.2 & 2.3). Our results are in line with previous studies showing that the link between GCs or body condition and reproductive success are detectable primarily under challenging conditions (e.g., Ouyang et al. 2015, Henderson et al. 2017, Vitousek et al. 2018a). They also extend these results by suggesting that the redox status is an important physiological system for reproductive performance when environmental conditions are not optimal. Although limitations in sample sizes especially in the 'good' year (2015) could have reduced our statistical power to identify meaningful relationships, our results suggest that physiological adjustments of individuals to challenging conditions rather than their physiological state under benign situations predict components of fitness, like reproductive success. This finding makes intuitive sense, since responding to fluctuations in external and internal conditions and mediating appropriate phenotypic changes is the one of the main functions of physiological signals (e.g., Martin et al. 2011). The observed relationships between physiological traits and fitness only in the year with 'bad' weather raise the question of whether these links are based on the mean value of a physiological trait or on responses, i.e. changes in physiological trait values along environmental and internal variation (i.e., the intercept and slope in a reaction norm model; Nussey et al. 2007; Dingemanse et al. 2010; Hau & Goymann 2015, Hau et al. 2016). In short-lived species like great tits that generally have one breeding event per year and live in predictable seasonal environments that undergo additional unpredictable climatic variations, the physiological responses to that variation may be particularly important to maximize reproductive outcomes.

In our study, the relationships between physiological traits and fitness proxies observed in the 'bad' year were sex-specific (Figures 2.2 & 2.3). Females that had a better oxidative status (i.e., high GPX and low ROM concentrations) under the more challenging conditions of 2016 fledged more and heavier offspring than females in a poor oxidative state. In males, traits other than oxidative status predicted reproductive success, with better body condition, high baseline and low stress-induced GCs being associated with more and heavier fledglings. Sex-specific relationships of GCs and reproductive success under challenging environmental conditions have previously been described in other bird species. For example, female blue-footed boobies (*Sula nebouxii*) had

higher baseline GC concentrations in the year when all nests failed due to the harsh environmental conditions associated with an 'El Niño' event compared to males, and compared to the subsequent year when environmental conditions were better (Wingfield et al. 1999). Similarly, in a three-year study female tree swallows had higher baseline GC concentrations and fledged fewer offspring only in the year with lowest ambient temperatures and reduced food availability, while this relationship was not present in males or in years with milder weather (Vitousek et al. 2018a). This supports the idea that the adrenocortical axes of females and males respond differently to natural conditions. Based on the results of the present study, ecological conditions may have a profound and sex-specific effect on the relationship between oxidative status and reproductive success.

As expected, physiological traits of the parents were associated with ambient temperature and cumulative rainfall. However, the only trait that showed a link to environmental conditions in both females and males was the redox system. GCs and body condition were related to climatic conditions just in males (Figure 2.4; Supplementary information 9.1, Table 5 & 6). Hence, despite experiencing similar environmental conditions male and female great tits showed sex-specific relationships in physiological traits with weather variables; males appeared more responsive than females because more male traits were associated with environmental conditions. It is worth noting that in females weather was primarily associated with oxidative status, which is the physiological system that also correlated with reproductive success, while in males more traits (GCs, redox system, body condition) were related to environmental conditions and reproductive success. Divergent physiological strategies may allow females and males to properly attune their internal state with life history decisions such as how much to invest in their current offspring. Future experimental studies, ideally using a repeated measures design will be important to probe whether changes in physiological traits of females and males in response to environmental conditions during the breeding season predict fitness. Since we only recorded two weather variables, we cannot rule out the effect of other, unmeasured environmental variables on reproductive success in our study. In blue tits (Henderson et al. 2017), nests that were more synchronous with the caterpillar peak (the main food source for nestling tits during the breeding season; Royama 1970) had more and heavier chicks than offspring from nests that were more asynchronous (hatched too early or too late), and this correlation was present in all study years, irrespective of weather conditions. Therefore, food abundance or quality may have influenced the reproductive outcome of the parents, contributing to explaining the difference in fledgling mass observed between years.

2.6. Conclusions

GCs, oxidative state parameters and body condition did not covary in males and females of a wild bird population studied over two years, despite evidence from previous studies suggesting that these physiological traits interact with each other (e.g., Haussmann & Marchetto 2010, Costantini et al. 2011a). As predicted, only in the year with the more challenging environmental conditions a relationship between physiological traits and reproductive success was present in free-living great tits, and these relationships were sex-specific. Relationships between physiological traits and environmental conditions also differed between sexes, with males appearing to be more responsive than females. Overall, our results support the view that relationships between physiological traits and fitness proxies in wild populations may only be detectable in some study years, suggesting that adjustments in physiological traits to challenging external circumstances may be more relevant for fitness than traits expressed under mild conditions.

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Authors' contributions

LM and MH designed the study. LM conducted the field work, run the corticosterone analysis in the lab, analyzed the data and drafted the manuscript. LM and SC conducted the oxidative status lab analysis. SC and MH contributed to manuscript preparation. All authors approved of the final version of the manuscript.

Permits

All experimental procedures were conducted according to the legal requirements in Germany and were approved by the governmental authorities of Oberbayern, Germany.

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Supporting information

The following Supporting Information is available for this article:

- Supplementary Information 9.1: tables.
- Supplementary Information 9.2: figure.

Chapter 3

Female variation in allocation of steroid hormones, antioxidants and fatty acids: a multilevel analysis in a wild passerine bird

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3.1. Abstract

The environment where an embryo develops can be influenced by components of maternal origin, which can shape offspring phenotypes and therefore maternal fitness. In birds that produce more than one egg per clutch, females differ in the concentration of components they allocate into the yolk along the laying sequence. However, identification of processes that shape female yolk allocation and thus offspring phenotype still remains a major challenge within evolutionary ecology. A way to increase our understanding is by acknowledging that allocation patterns can differ depending on the level of analysis, such as the population *versus* the among-female (within-population) level. We employed mixed models to analyze at both levels the variation in allocation along the laying sequence of four steroid hormones, three antioxidants, and four groups of fatty acids present in the egg yolks of wild great tits (*Parus major*). We also quantified repeatabilities for each component to study female consistency. At a population level, the concentrations/proportions of five yolk components varied along the laying sequence, implying that the developmental environment is different for offspring developing in first *versus* last eggs. Females varied substantially in the mean allocation of components and in their plasticity along the laying sequence. For most components, these two parameters were negatively correlated. Females were also remarkably repeatable in their allocation. Overall, our data emphasize the need to account for female variation in yolk allocation along the laying sequence at multiple levels, as variation at a population level is underpinned by different individual patterns. Our findings also highlight the importance of considering both levels of analysis in future studies investigating the causes and fitness consequences of yolk compounds. Finally, our results on female repeatability confirm that analyzing one egg per nest is a suitable way to address the consequences of yolk resource deposition for the offspring.

3.2. Introduction

Female birds can influence the physiological conditions in which their embryos will develop by differential allocation of resources into the egg yolk, thus generating variation in offspring phenotype and influencing fitness (Mousseau and Fox 1998). Such resources can be hormones (Schwabl 1993) and nutrients (Surai 2002, Hulbert and Abbott 2011), which can affect embryonic growth and development and also provide protection to oxidative damage. In bird species that produce more than one egg per clutch, females often vary the concentration of components they allocate along the laying sequence (e.g., Royle et al. 1999, Blount et al. 2002, Hõrak et al. 2002, Saino et al. 2002, Tschirren et al. 2004, Bourgault et al. 2007, Rubolini et al. 2011, Lessels et al. 2016, Toledo et al. 2016). Hence, depending on the egg in which they develop, offspring from the same clutch can be exposed to different environments during embryonic development. Despite maternal effects being an important factor in evolution (Mousseau and Fox 1998), the identification of the processes that shape female yolk allocation still remains a major challenge in biology.

A way to increase our understanding of yolk allocation is by acknowledging that females can show different patterns of allocation along the laying sequence when analyzed at multiple levels (Meyers and Bull 2002). This phenotypic plasticity (defined as the property of a given genotype to produce different phenotypes as environmental conditions change; Via et al. 1995, Pigliucci 2001, Nussey et al. 2007) observed along the laying sequence can be analyzed at a population level by looking at the average female allocation of components along the laying sequence (Figure 3.1a). At a population level, consistent patterns of average yolk allocation along the laying sequence have been reported for several bird species. For example, domestic canaries (*Serinus canaria*) and black-headed gulls (*Chroicocephalus ridibundus*) increase the concentration of androgens, while zebra finches (*Taeniopygia guttata*) decrease the concentration of these components from the first to the last egg (reviewed by Groothuis et al. 2005, Gil 2008). This population-level variation, hereafter referred to as "mean phenotypic plasticity", has been interpreted in light of sibling competition and parent-offspring conflict (Müller et al. 2007). However, a lack of consistency across populations of the same species has also been reported, for example in great tits (*Parus major;* Tschirren et al. 2004, Groothuis et al. 2008, Lessels et al. 2016). This inconsistency indicates that the patterns of allocation are more complex than previously assumed.

Phenotypic plasticity in allocation patterns at a population level might not necessarily provide information regarding individual-level variation (Nussey et al. 2007, Dingemanse et al. 2010). The same pattern observed at a population level might be driven by females differing in the mean allocation of components (Figure 3.1b), in the slope of changes along the laying sequence (hereafter referred to as "individual phenotypic plasticity"; Figure 3.1c), or in both parameters which covary (Figure 3.1d). Mixed results across populations of the same species may therefore result from differences among females in allocation patterns within each population (Nussey et al. 2007, Dingemanse et al. 2010). Individual female variation can be quantified by using a reaction norm approach, which allows us to estimate how much of each yolk component females transfer on average into the yolk (i.e., the elevation of the reaction norm), the change in female allocation along the laying sequence (i.e., individual phenotypic plasticity), as well as the covariation between elevation and slope (Nussey et al. 2007, Dingemanse et al. 2010, Dingemanse and Wolf 2013). In particular, the presence of correlations between the mean allocation of components and the individual phenotypic plasticity suggests that maternal effects cannot be fully evaluated by studying each of these components separately, since selection could be acting on each of these sources of variation and/or directly on their correlation. Since natural selection operates at the individual level, accounting for individual female variation (i.e., mean allocation, phenotypic plasticity and the correlation between these two parameters) in yolk allocation along the laying sequence is therefore of key importance if we aim to understand the evolutionary causes and consequences of variation in female allocation.

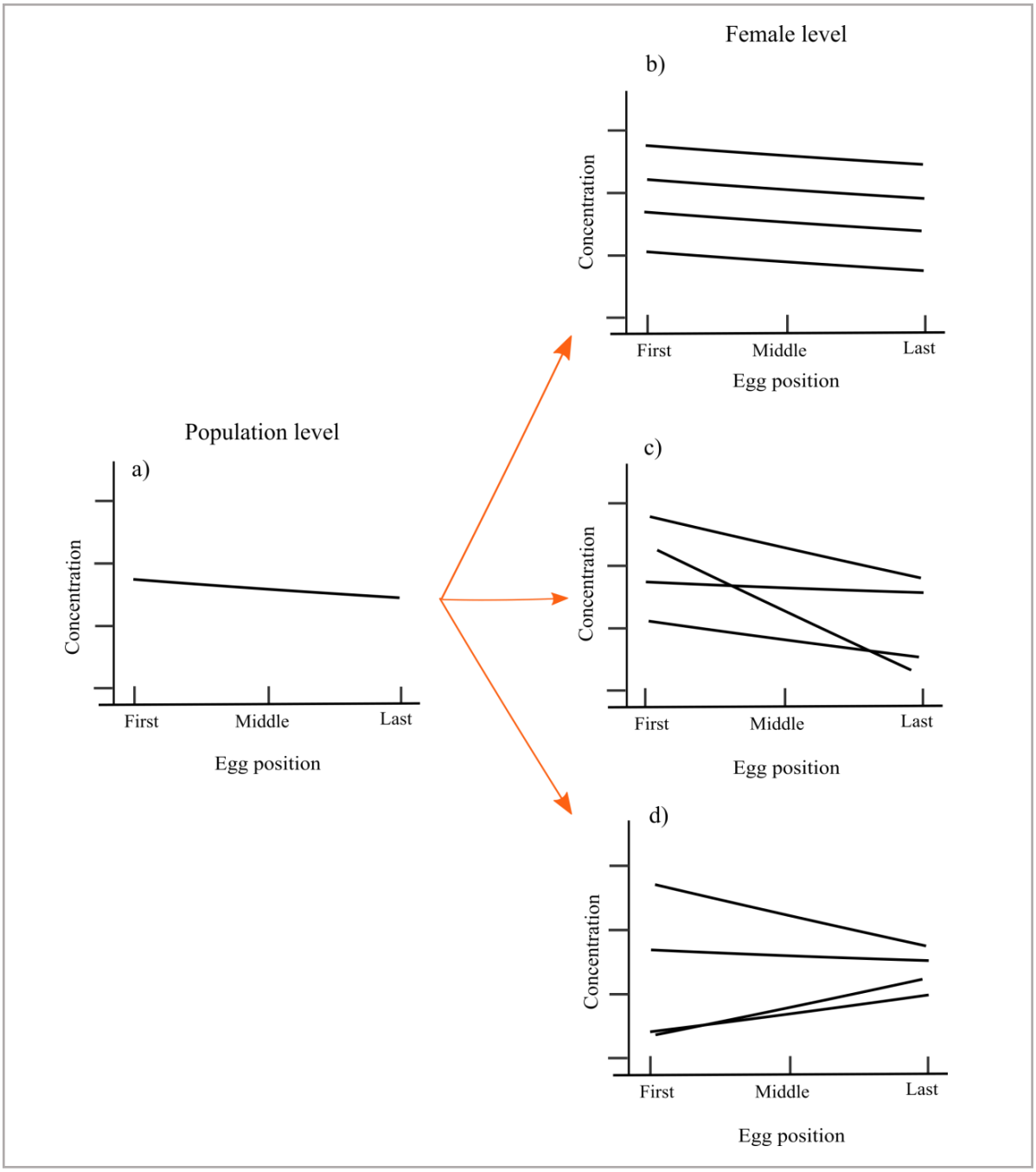

Figure 3.1. Schematical depiction of the two levels of analysis used in this study to understand female yolk allocation in avian species that produce clutches of more than one egg. (a) The mean phenotypic plasticity (i.e. average population variation) along the laying sequence can be caused by variation among females. (b) Females (individuals represented by different solid lines) can vary in the mean allocation of yolk components, (c) in the individual phenotypic plasticity (i.e. slope of allocation along the laying sequence), or (d) in both the mean and slope of allocation. In the latter case, the two parameters can also be positively or negatively correlated. For example, females that on average allocate a high concentration of a specific yolk component will more strongly decrease the concentration of that component along the laying sequence ((d); upper line).

Here we employed linear mixed models to study the allocation along the laying sequence at both population- and among female-levels in the yolks of 11 clutches of freshly laid eggs collected from a wild population of great tits. Our emphasis in this investigation was to provide new insights into individual-level patterns. Furthermore, since the majority of research to date has focused on androgens, here we also aim at simultaneously quantifying additional yolk components to increase our understanding of the factors contributing to such differences in allocation patterns observed along the laying sequence. For each egg collected we measured the concentrations of four steroid hormones (the androgens androstenedione, 5α-dihydrotestosterone, testosterone, and the glucocorticoid corticosterone), three antioxidants (vitamin E, lutein, zeaxanthin) and the proportions of four groups of fatty acids (saturated fatty acids, monounsaturated fatty acids, omega (ω)-3 polyunsaturated fatty acids (PUFA), ω-6 PUFA). We then quantified female consistency in allocation for each yolk component (e.g., whether females that allocate on average high levels of a yolk component always allocate high concentrations along the laying sequence compared to other females) by calculating adjusted repeatabilities.

We selected specific yolk components for our analysis because of possible interactive effects on offspring phenotype, although the statistical determination of such possible interactions is beyond the scope of the current study. Steroid hormones such as androgens and glucocorticoids can enhance offspring growth, competitive ability and survival, while also possibly causing immunosuppression and oxidative stress (Schwabl 1993, Groothuis and Schwabl 2002, Groothuis et al. 2005, Gil 2008, Groothuis and Schwabl 2008, Haussmann et al. 2012, Treidel et al. 2013). Conversely, antioxidants like the carotenoids and vitamin E can enhance the immune system and mitigate oxidative stress caused by embryo growth (Surai 2000, Saino et al. 2003, Yigit et al. 2014, Parolini et al. 2017, Watson et al. 2018). Fatty acids, in turn, provide the avian embryo with almost all the energy and building blocks required to sustain development within the egg (Noble and Cocchi 1990, Surai and Speake 2008). In particular, PUFAs are vital components for the formation of cell membranes, heart functioning and brain development (Hulbert and Abbott 2011). However, highly unsaturated PUFAs are susceptible to lipid peroxidation through reactive oxygen species that are generated by embryonic metabolism (Pamplona et al. 2002, Larsson et al. 2004, Hulbert and Abbott 2011, Yigit et al. 2014). Furthermore, we recorded lay date, ambient temperatures at the time of laying, and female body condition. Factors such as lay date and ambient temperature may explain the concentration/proportion of some yolk components, especially those known to be

highly influenced by the quality and quantity of food consumed by the mother (antioxidants like vitamin E and carotenoids and PUFAs; Surai and Speake 2008; Hulbert and Abbott 2011). Internal variables such as female body condition are also usually considered important factors for determining the yolk allocation of substances like hormones (reviewed by Groothuis et al. 2005), antioxidants (Blount et al. 2002, Williamson et al. 2006) and fatty acids (Raclot 2003, Price et al. 2008).

3.3. Materials and methods

3.3.1. Study species, field site and sampling

Great tits are small passerine birds that breed inside cavities. Females usually lay one egg each day early in the morning. While having a seed-dominated winter diet, great tits feed primarily on caterpillars during the breeding season (Royama 1970) which are an important source of proteins and fatty acids (Isaksson and Andersson 2007, Andersson et al. 2015, Isaksson et al. 2015).

For this study, fresh laid eggs of entire clutches were collected in April and May 2015 from a nest box population of great tits in the Dellinger Buchet, a mosaic of deciduous and coniferous forest in Southern Germany (Bavaria; 48˚03’ N, 11˚13’ E, 620 m above sea level). The breeding stage was monitored every second day from the first signs of nest construction onwards. Eggs were collected on the date of lay between 8:00 and 13:00h and each removed egg was replaced with a dummy egg. In total, 93 eggs from 11 first clutches were collected. Clutch sizes ranged from 6 to 10 eggs (mean ± SD: 8.45 ± 1.13 eggs). Once in the laboratory, egg measurements were taken following established protocols by Lessells et al. (2002). Briefly, eggs were weighed, opened, and the yolk separated from the remainder of the egg. Excess albumen was removed from the yolk by rolling it on a piece of paper. During this step the vitelline membrane of the yolk got disrupted in three out of 93 eggs, but since the amount of yolk lost was minor, we included these eggs in the analysis. The yolk was weighed, homogenized in distilled water and immediately stored at -80˚C until further analysis. The egg shell was washed, weighed and dried at room temperature until the next morning when the dry shell was weighed again. Albumen mass was estimated by subtracting the wet yolk mass and the dry shell mass from the total egg weight.

We measured local environmental conditions during the formation of the eggs. Ambient temperature was recorded every hour via i-buttons (DS9093A+ Thermochron iButton) placed in

12 different places across the study site. For our analyses, we used the mean temperature on the three days preceding the lay date of each egg, hereafter referred as mean temperature, since the phase of follicular growth lasts about three days in great tits (Walsberg 1983). Preliminary exploration of our data showed that other environmental factors like rainfall did not have an effect on the allocation of yolk components (results not shown). Hence, we only included mean ambient temperature in our analyses.

Nine of the 11 females were captured when incubating the replaced eggs, on average 4.78 ± 1.62 days after they had laid their last egg. Females were marked with a numbered aluminum ring and plastic split rings with a unique color combination for individual identification. All females were adults (> 1 year), as determined from plumage characteristics. Body mass (to the nearest 0.1 g), tarsus length (to the nearest 0.1 mm) and wing length (to the nearest 0.1 mm) were measured for each captured individual. Scaled body mass index was used as an indicator of female condition, since it accounts for the allometric relationship between different measures of body size and mass (Peig and Green 2009). As recommended by Peig and Green (2009), parameters with the highest correlation in our population were used to estimate female condition: body mass and wing length (r = 0.68; p-value = 0.05).

3.3.2. Steroid hormone analysis

To quantify androstenedione, 5α-dihydrotestosterone, testosterone, and corticosterone concentrations, we conducted radioimmunoassays following the method described by Wingfield and Farner (1976), modified by Goymann et al. (2008) with additional adjustments for the measurement of egg yolk following Schwabl (1993). Steroids were extracted from the yolk in two sets of assays. Because one of the key aspects of our manuscript was to study yolk allocation of individual females, and variation in yolk allocation among females is typically higher than within females (reviewed by Groothuis et al. 2005), eggs from the same clutch were always included in the same assay. On average, 50 µl of the yolk/water emulsion was transferred to 16 x 100 glass test tubes. Along with the samples, two blanks containing 300 µl distilled water and three positive controls containing 100 µl stripped chicken plasma pools were also prepared. Distilled water was added to all tubes to have the same final volume (300 µl). Next, we added 10 µl tritiated steroid (1500 dpm; PerkinElmer, MA, USA) of all steroid hormones to be measured to all tubes except the blanks to estimate extraction efficiency. We then added 4 ml of diethyl ether to each sample.

After overnight equilibration the samples were centrifuged, the supernatant was collected and dried under a nitrogen stream in a water bath at 40 ºC. Each sample was then subjected to a second extraction by adding 2 ml dichloromethane. The dried supernatant was re-suspended in 1 ml 99% ethanol. After an overnight reconstitution, extracts were centrifuged, the supernatant collected and again dried under a nitrogen stream in a water bath at 40 ºC, and reconstituted in isooctane with 2% ethyl acetate. Steroids were then further separated via diatomaceous earth column chromatography. Fractions containing different steroids were eluted by mixing ethyl acetate (EtAc) with isooctane at increasing concentrations (2%, 10%, 25%, and 45% EtAc for androstenedione, 5α-dihydrotestosterone, testosterone and corticosterone, respectively). Each eluted fraction was collected in 12 × 75 mm glass tubes and evaporated under a nitrogen stream. Androgens (androstenedione, 5α-dihydrotestosterone, and testosterone) were re-dissolved in 300 µl phosphate-buffered saline. 80 µl of the resuspended fraction were used to estimate individual extraction recoveries. Recoveries for the two sets of columns (n = 50 samples per set) were within the expected range previously reported for great tits (Tschirren et al. 2004, Groothuis et al. 2008, Lessels et al. 2016) and mean ± SD were as follows: androstenedione = 83 ± 2.83%, 5α-dihydrotestosterone = 55% (in both sets), testosterone = 51 ± 1.41%. Duplicates of 100 µl were used for the radioimmunoassays. The hormone concentration of each sample was corrected for the individual extraction efficiency. Some of the samples had concentrations above the upper detection limit of the assay (which was at 200 pg per tube), hence we extracted another proportion of the yolk following the same protocol but using a smaller volume (70 µl) of the extracted sample for the radioimmunoassays (androstenedione: 79 samples; testosterone: 6 samples). Recoveries for these two additional sets of columns were comparable to the first set of assays (mean ± SD only for androstenedione): androstenedione = 78.5 ± 0.71%, testosterone = 89%. Polyclonal antibodies used were the following: AN6-22 for androstenedione, DT3-351 for 5α-dihydrotestosterone and T3-125 for testosterone (all Esoterix Endocrinology, Inc., CA, USA). The lower detection limit was 0.80 pg/ml for androstenedione, 0.56 pg/ml for 5α-dihydrotestosterone, and 0.36 pg/ml for testosterone. Blanks were all below detection limits. All samples were analyzed in two assays. The intra-assay variations for both assays, determined from the three positive controls, were: androstenedione = 7.65 ± 0.85%, 5α-dihydrotestosterone = 4.15 ± 0.25%, testosterone = 9.3 ± 0.5%. The inter-assay variations, determined by including the first positive control per assay, were (mean ± SD): androstenedione = 16.2%, 5α-dihydrotestosterone = 3.4%, and testosterone = 12.4%.

For those samples that needed to be re-extracted the intra-assay variations were: androstenedione (mean ± SD) = 8.65 ± 1.13%, testosterone = 4.1%. Corticosterone concentrations were determined by enzyme immunoassay (Lot No: 12041402D and 08241511, Enzo Life Sciences GmbH, Germany). After column chromatography, the corticosterone fractions of the samples were dried and then dissolved in 350 µl assay buffer. An aliquot of 80 µl was used to estimate individual extraction recoveries (mean ± SD recoveries were 57 ± 11.31 %). Duplicates of 100 µl were added to individual wells and samples were distributed across 4 assays. The intra-assay coefficients of variation were 1.9%, 5.5%, 2.4%, 2.3% (calculated from two replicate standards per plate) and the inter-assay variation was 3%. The corticosterone antibody has a 0.5% cross-reactivity with progesterone, but the column chromatographic separation of steroid hormones had separated corticosterone from progesterone (Wingfield and Farner 1976), so that cross-reactivity can be excluded.

3.3.3. Antioxidant extraction and HPLC analysis

Vitamin E (α-tocopherol) and carotenoids (lutein and zeaxanthin) were extracted simultaneously. Briefly, to 20 mg yolk, 200 µl acetone with internal standard - 600 µM retinyl acetate and 1 mM tocopheryl acetate (Sigma-Aldrich, Stockholm, Sweden) was added, followed by vortexing. The samples were then left overnight at -80ºC. The following day, 200 µl tert-butyl methyl ether was added, followed by vortexing. Samples were centrifuged at 10ºC for 5 min (13,000 rpm), and the supernatant was transferred to a new tube and dried under nitrogen gas. The samples were washed twice with 200 µl acetone, followed by vortexing and centrifugation; the supernatant was removed to a new tube between the washes. Again, samples were dried under nitrogen gas. The residue was dissolved in 100 µl methanol-acetonitrile (30:70). The amount of α-tocopherol, lutein and zeaxanthin were determined by high performance liquid chromatography (HPLC) with the following specifications: column Phenomenex Syndergi 4u Hydro-RP 80A, 250 × 3 mm + 4 × 2 mm guard column, isocratic 20 % MeOH, 80 % AcCN, 12 min, 1,2 ml/min, oven 40 °C, injection 5 µl, UV 450 nm, FL ex: 290 nm, em: 325 nm. The concentrations were calculated from standard curves made from lutein, zeaxanthin and tocopherol, along with corrections for their respective internal standards.

3.3.4. Fatty acid extraction and quantification

The fatty acids were extracted as described by Eikenaar et al. (2017). Briefly, a total lipid extraction of approximately 5 mg of yolk was done using chloroform and methanol (2:1 v/v). Base methanolysis was carried out to transform the fatty acids into corresponding fatty acid methyl esters (FAMEs). The FAMEs were extracted using heptane (>99%; VWR Prolabo), and the extracts were analyzed using an Agilent 5975 mass spectrometer coupled to an Agilent 6890 gas chromatograph with an HP-INNOWax PEG column (30 m, 0.25 mm i.d., 0.25 mm film thickness; Agilent). Analyses and quantification of chromatograms were performed using ChemStation software (Agilent). FAMEs were identified by comparing mass spectra and retention times with those of synthetic standards (Supelco 37-Component FAME Mix, Sigma-Aldrich).

3.3.5. Data handling and statistical analysis

In total, we studied 11 yolk components. For yolk steroid hormones and antioxidants, the statistical analyses were done using the concentration (in pg/ml or standard micromolar, respectively), while for fatty acids we analyzed the proportion of each fatty acid group (Andersson et al. 2015, Isaksson et al. 2017). For simplicity, lutein and zeaxanthin were pooled and referred to as total carotenoids whenever both components showed similar results. Fatty acid proportions were calculated by dividing the peak area of each fatty acid by the sum of the peak areas of all fatty acids in each individual sample (Andersson et al. 2015, Isaksson et al. 2017). The proportions of all individual fatty acids within a certain chemical class of fatty acid were then combined to obtain relative levels of total saturated fatty acids, total monounsaturated fatty acids, total ω-3 PUFA, and total ω-6 PUFA (Andersson et al. 2015, Isaksson et al. 2017). Furthermore, one of our aims was to study the difference in yolk components along the laying sequence. Since great tits vary in clutch size, analyzing yolk components in terms of only egg number is inadequate. We therefore used the relative egg position (determined as egg position/N eggs per clutch) within a range from 0 to 1 as a standardized variable in our analysis (indicated as ‘first’, ‘middle’ and ‘last’ in the figures for illustrative purposes only).

We ran three univariate mixed-models fitting each yolk component or group of yolk component, respectively, as a response variable. All continuous explanatory variables were mean-centered and their variance standardized to facilitate comparison of variance components across traits. First, to study the relationship between yolk components and female body condition we used the scaled

body mass index as a measure of female condition, egg position, and mean ambient temperature (temperature range = 7.6 – 16.8ºC) as covariates. Lay date was not included in this analysis to avoid overparametrization. Despite the fact that we were interested in the effect that female body condition has on yolk components (i.e., fixed factor), female identity and the interaction between female identity and laying sequence were fitted in the model as random elevation and random slope, respectively (for a further discussion of the rationale of this approach see Schielzeth and Forstmeier, 2009).

Second, to study female allocation at the population and individual level, we used a random regression model (i.e., a reaction norm framework; Nussey et al. 2007, Dingemanse et al. 2010). Egg position, lay date (date of first egg, range = 16th of April – 9th of May) and mean ambient temperature were fitted as covariates (the correlation between laying date and mean ambient temperature was relatively low; r = 0.26; p-value = 0.01). Female identity (i.e., random elevation) and the interaction between female identity with respect to laying (i.e., random slope) were also fitted in the model. By using this random elevation-slope approach we were able to estimate three parameters: i) the among-female variation in the average concentration/proportion of egg components (i.e., variance in elevation; Figure 3.1b), ii) the among-female variation in plasticity along the laying sequence (i.e., variance in slope; Figure 3.1c), and iii) the correlation between elevation and slope (Figure 3.1d).

Finally, to calculate the repeatability (R) of yolk components among females, we built a model similar to the one described in the previous paragraph, but only including female identity as a random intercept. The repeatability of female yolk allocation was calculated as the variance component explained by female identity divided by the total variance (female identity + residual) in the presence of fixed effects ("adjusted repeatability"; Nakagawa and Schielzeth 2010; i.e., egg position, lay date and mean ambient temperature).

All statistical analyses were performed in R statistical freeware R-3.3.3 (R Core Team 2017) using the "lme4" and "arm" packages in a Bayesian framework with non-informative priors. We assumed a Gaussian error distribution, which was confirmed for all response variables after visual inspection of model residuals. When necessary, response variables were transformed (details on transformations are provided in Supplementary information 9.3). We subsequently used the *sim* function to simulate values from the posterior distributions of model parameters. We

extracted the 95% Bayesian Credible Interval (CrI) around the mean (Gelman and Hill 2007), and assessed statistical support by obtaining the posterior distribution of each parameter. CrI provide more valuable information than p-values, like for example, the uncertainty around the estimates. We use the term "meaningful effect" if zero was not included within the 95% CrI (Korner-Nievergelt et al. 2015). For intervals overlapping zero only slightly, we report the posterior probability of the estimate being positive or negative (see Korner-Nievergelt et al. 2015 for further discussion of how to infer conclusions from Bayesian statistical analysis).

3.4. Results

3.4.1. Egg yolk components, environmental effects and female body condition

Egg mass increased along the laying sequence by 12%, while yolk mass only tended to increase (Supplementary information 9.3, Table 1). The concentrations of androstenedione, 5α-dihydrotestosterone and testosterone were substantially higher than those of corticosterone (Supplementary information 9.3, Table 2). Among the antioxidants, the carotenoid lutein was the most abundant, followed by zeaxanthin and vitamin E. Furthermore, 20 fatty acids were identified (Supplementary information 9.3, Table 3), with the monounsaturated fatty acid group contributing most to the total fatty acid content (around 46%; Supplementary information 9.3, Table 2), followed by saturated fatty acids, ω-6 PUFAs and lastly ω-3 PUFAs.

Females that laid eggs later in the breeding season allocated higher concentrations of androstenedione and higher proportions of saturated fatty acids and ω-3 PUFAs into the yolk, while the concentration of corticosterone and the proportion of ω-6 PUFAs decreased over the breeding season (Table 3.1). The mean ambient temperature had a positive effect on the proportion of ω-6 PUFAs, and a negative one on the concentrations of corticosterone and the proportion of monounsaturated fatty acids (Table 3.1). Neither lay date nor mean ambient temperature influenced the concentrations of yolk antioxidants. Female scaled body mass index had a negative effect on 5α-dihydrotestosterone concentrations and a positive one on vitamin E concentrations in the yolk (Supplementary information 9.3, Table 4). However, scaled body mass index had a weak effect, if any, on most of the other yolk components.

3.4.2. Mean phenotypic plasticity in yolk components along the laying sequence: population level

5α-dihydrotestosterone, all three antioxidants (vitamin E and both carotenoids), the proportion of ω-6 PUFA and the ratio of total ω-6/ total ω-3 PUFA decreased over the laying sequence (Table 3.1; Figure 3.2). The other steroid hormones (androstenedione, testosterone, and corticosterone) and the proportion of ω-3 PUFA did not change, while the proportion of saturated fatty acids increased from the first to the last egg. There was moderate support for the proportion of monounsaturated fatty acids to increase over the laying sequence (posterior probability = 0.97).

	Steroid hormones[a]				**Antioxidants[b]**				**Fatty acids[c]**				
	A4	DHT	Testo	Cort	Vit E	Lut	Zea	Carot	SFA	MUFA	ω-3 PUFA	ω-6 PUFA	ω-6/ω-3 PUFA
Fixed factors β (95% CrI)													
Intercept	3.04 (2.92; 3.17)	2.51 (2.34; 2.67)	22.45 (18.35; 26.54)	0.17 (0.14; 0.19)	1.08 (0.91; 1.25)	10.58 (8.51; 12.59)	2.28 (1.79; 2.75)	10.92 (8.79; 12.99)	-0.95 (-1.00; -0.89)	-0.16 (-0.18; -0.14)	-3.31 (-3.53; -3.08)	-1.27 (-1.40; -1.15)	1.83 (1.52; 2.13)
Egg position[d]	0.01 (-0.05; 0.08)	**-0.08 (-0.14; -0.01)**	0.78 (-0.71; 2.28)	-0.00 (-0.02; 0.02)	**-0.14 (-0.22; -0.07)**	**-1.29 (-2.11; -0.47)**	**-0.22 (-0.42; -0.02)**	**-1.30 (-2.13; -0.45)**	**0.02 (0.00; 0.05)**	0.01 (-0.00; 0.02)	0.03 (-0.01; 0.08)	**-0.05 (-0.08; -0.02)**	**-0.07 (-0.13; -0.01)**
Lay date[e]	**0.13 (0.01; 0.24)**	0.07 (-0.08; 0.23)	0.33 (-3.67; 4.38)	**-0.03 (-0.06; -0.01)**	0.00 (-0.16; 0.17)	1.28 (-0.73; 3.30)	0.18 (-0.30; 0.67)	1.38 (-0.71; 3.45)	**0.07 (0.02; 0.13)**	-0.02 (-0.04; 0.01)	**0.26 (0.04; 0.48)**	**-0.16 (-0.29; -0.04)**	**-0.38 (-0.69; -0.08)**
Mean ambient temperature[f]	0.02 (-0.04; 0.08)	0.03 (-0.01; 0.08)	1.17 (-0.56; 2.91)	**-0.02 (-0.04; -0.00)**	0.05 (-0.03; 0.13)	0.37 (-0.57; 1.28)	0.08 (-0.14; 0.30)	0.37 (-0.56; 1.29)	0.00 (-0.01; 0.01)	**-0.01 (-0.02; -0.00)**	-0.02 (-0.04; 0.01)	**0.01 (0.00; 0.03)**	0.03 (-0.01; 0.06)
Random factors σ^2 (95% CrI)													
Among-female variance													
V elevation[g]	0.04 (0.02; 0.07)	0.07 (0.05; 0.10)	39.71 (23.26; 64.44)	0.00 (0.00; 0.00)	0.06 (0.03; 0.11)	9.44 (5.10; 15.86)	0.54 (0.29; 0.94)	10.23 (5.27; 17.69)	0.01 (0.01; 0.01)	0.00 (0.00; 0.00)	0.04 (0.04; 0.06)	0.13 (0.11; 0.19)	0.25 (0.22; 0.38)
V slopes[h]	0.01 (0.01; 0.02)	0.01 (0.01; 0.03)	11.13 (8.88; 19.71)	0.00 (0.00; 0.00)	0.02 (0.02; 0.03)	2.82 (2.28; 4.87)	0.16 (0.13; 0.29)	3.05 (2.55; 5.29)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.01 (0.00; 0.02)	0.02 (0.01; 0.05)	0.04 (0.03; 0.11)
Cor elevation-slopes[i]	**-0.85 (-0.96; -0.65)**	-0.21 (-0.56; 0.19)	**-0.89 (-0.97; -0.72)**	-0.08 (-0.56; 0.43)	**0.86 (0.68; 0.97)**	**-0.62 (-0.89; -0.17)**	**-0.87 (-0.97; -0.68)**	**-0.88 (-0.97; -0.69)**	-0.21 (-0.52; 0.15)	**-0.68 (-0.90; -0.31)**	-0.08 (-0.41; 0.29)	-0.15 (-0.44; 0.17)	-0.13 (-0.43; 0.22)
Residual variance	0.07 (0.05; 0.09)	0.03 (0.02; 0.04)	52.22 (38.93; 69.93)	0.004 (0.00; 0.01)	0.11 (0.09; 0.16)	14.58 (10.8; 19.65)	0.89 (0.65; 1.19)	15.42 (11.39; 20.81)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.01 (0.01; 0.01)	0.01 (0.01; 0.02)

Table 3.1. Results from linear mixed-effects models estimating fixed and random effects to explain variation in yolk components and variation among females. Egg position, lay date and mean ambient temperature were fitted as covariates, and random slopes were fitted for female identity with respect to egg position. We present fixed (β) and random (σ2) parameters with their 95% credible intervals (CrI) in brackets. All explanatory variables were mean centered; hence the intercepts refer to the average value of covariates. Fixed factors with a statistically meaningful effect (i.e., if zero is not included within the 95% CrI) are presented in bold. Estimates and CrI of '0.00' represent an effect smaller than 0.01.

[a] A4, androstenedione; DHT, 5α-dihydrotestosterone; Testo, testosterone; Cort, corticosterone.

A4 and DHT concentrations were log10 transformed.

[b] Vit E, vitamin E; Lut, lutein; Zea, zeaxanthin; Carot, sum of lutein and zeaxanthin.

All antioxidant concentrations were square root transformed.

[c] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA polyunsaturated fatty acids.

All fatty acid proportions were logit transformed; the total ω-6/total ω-3 PUFA ratios were log10 transformed.

[d] Egg number corrected by total clutch size.

[e] Date of first egg laid.

[f] Mean ambient temperature for the 3 days prior to the lay date of each egg.

[g] Total amount of variation in reaction norm elevation among-females.

[h] Total amount of variation in reaction norm slopes among-females.

[i] Elevation-slope correlation.

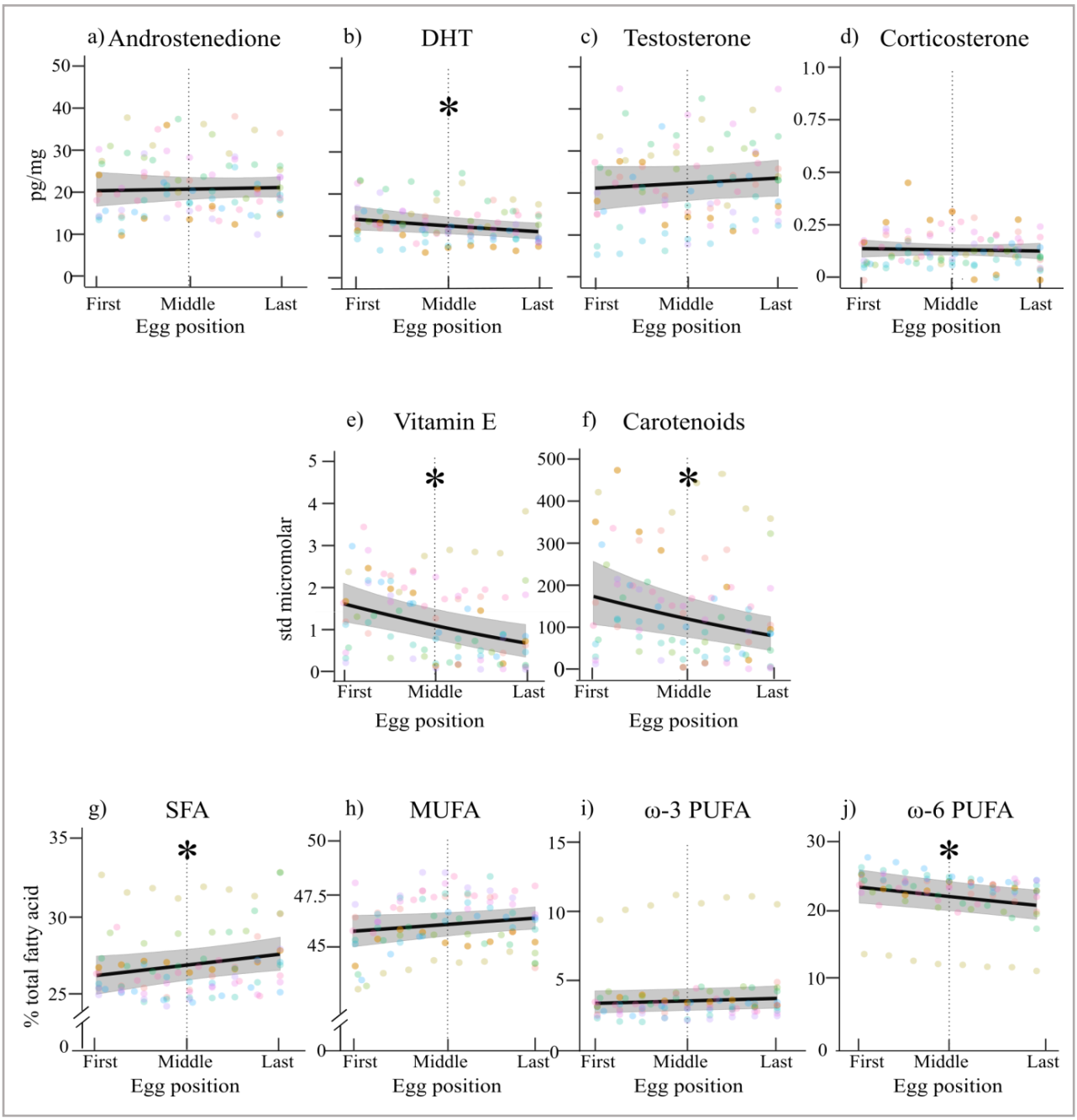

Figure 3.2. Mean phenotypic plasticity of steroid hormones (a–d), antioxidants (e–f), and fatty acids (g–j) along the laying sequence in great tit egg yolks. Egg position is provided as the egg number relative to total clutch size for each nest. Note that in all statistical analyses the laying sequence was included as a continuous variable and references in x-axis to egg position within the laying sequence are only for illustrative purposes. Filled circles show raw data for each egg; each color indicates a different nest. The black solid line represents average concentrations or proportions of components, with 95% credible intervals indicated in grey shading. (b) DHT, 5α-dihydrotestosterone; (e) vitamin E, α-tocopherol; (f) carotenoids, sum of lutein and zeaxanthin; (g) SFA, saturated fatty acids; (h) MUFA, monounsaturated fatty acids; (i–j) PUFA, polyunsaturated fatty acids. * Statistically meaningful support for an effect of 'egg position' on the yolk component concentration/proportion.

3.4.3. Variation in yolk components along the laying sequence: female level

Females not only differed in mean concentrations/proportions of yolk components allocated, but also in their phenotypic plasticity over the laying sequence (variation in elevation and slope, respectively; Table 3.1, Figure 3.3). Furthermore, the mean trait value and the slope were negatively correlated (i.e., showed a "fanning-in" pattern) in five of the 11 components measured: androstenedione, testosterone, both carotenoids (i.e., lutein and zeaxanthin) and monounsaturated fatty acids (Figure 3.3, Table 3.1). Females that on average allocated low levels of androstenedione, testosterone and monounsaturated fatty acids were more plastic, i.e., increased the concentrations/proportions of these yolk components along the laying sequence more strongly than did females with high average concentrations/proportions. In contrast, the decrease in carotenoid concentrations along the laying sequence was more pronounced in females that allocated higher average concentrations of carotenoids into their yolks. For vitamin E, the correlation between elevation and slope was positive (Table 3.1), but this correlation was driven by one female (Figure 3.3c; uppermost line) and therefore this result should be treated with caution. The elevation and slope of allocation for the rest of the components were only weakly correlated (Table 3.1).

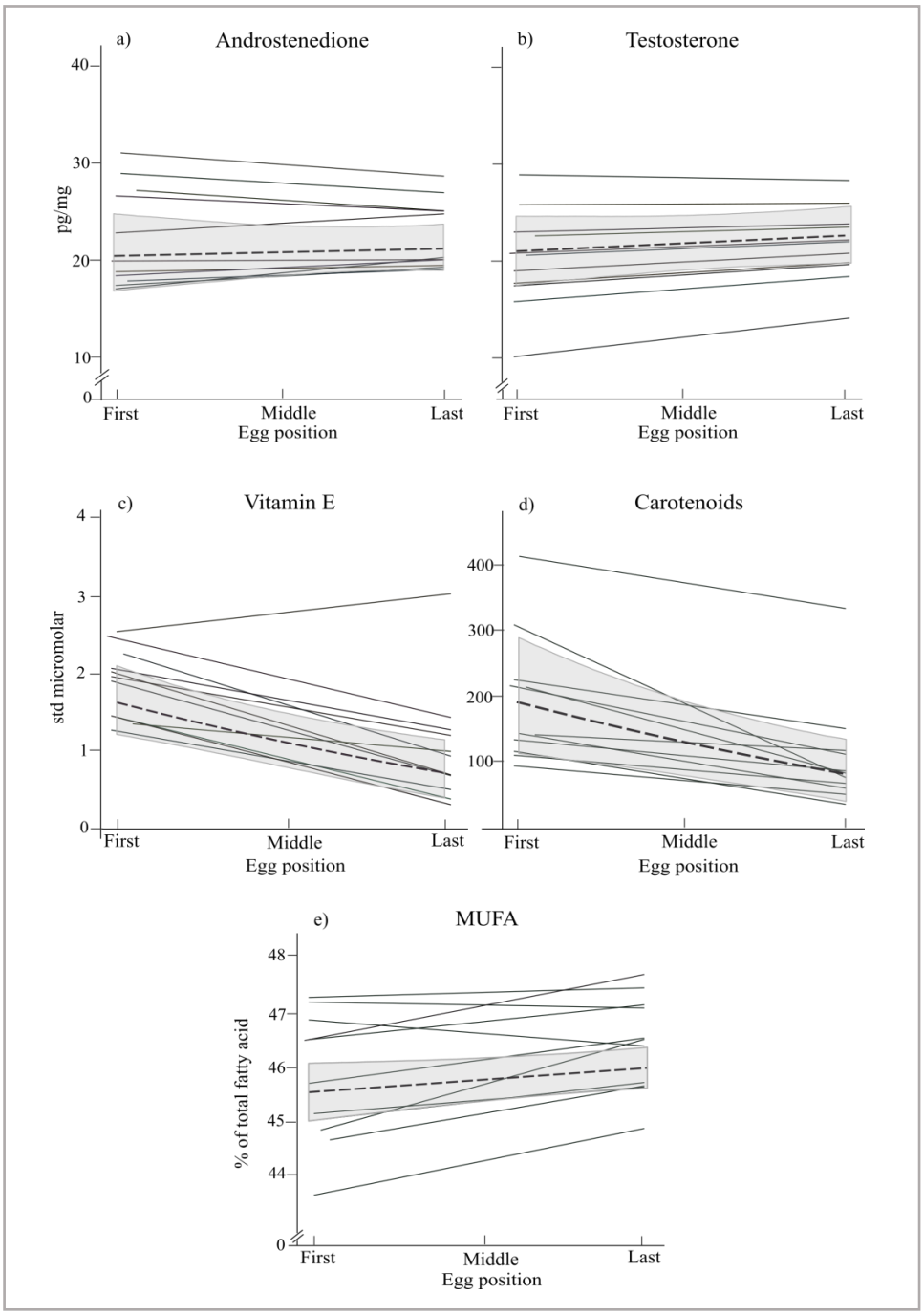

Figure 3.3. Reaction-norm plots of steroid hormones (a–b), antioxidants (c–d), and fatty acids (e), illustrating among-female variation in yolk allocation patterns along the laying sequence. Plots are shown only for those yolk components where elevation and slope of allocation n were correlated. Egg position is shown as the egg number relative to total clutch size for each nest. Note that in all statistical analyses the laying sequence was included as a continuous variable and references in x-axis to egg position within the laying sequence are only for illustrative purposes. Gray lines indicate different nests. Mean population concentrations or proportions of components (dashed black line) and 95% credible intervals (gray shading) are also shown as a reference. (c) Vitamin E, α-tocopherol; (d) carotenoids, sum of lutein and zeaxanthin; (e) MUFA, monounsaturated fatty acids.

For the vast majority of the yolk components (10 of 11), female adjusted repeatabilities were higher than 0.30 (Figure 3.4). The fatty acid groups had the highest repeatability values (ω-3 PUFA: R = 0.90; ω-6 PUFAs: R = 0.92), followed by the antioxidants, which all had values of repeatability approaching $R \sim 0.40$. Steroid hormones showed the widest range of repeatabilities, with 5α-dihydrotestosterone exhibiting a high (R = 0.64), testosterone and androstenedione a moderate (R= 0.43 and R = 0.33 respectively), and corticosterone a relatively low repeatability (R = 0.18).

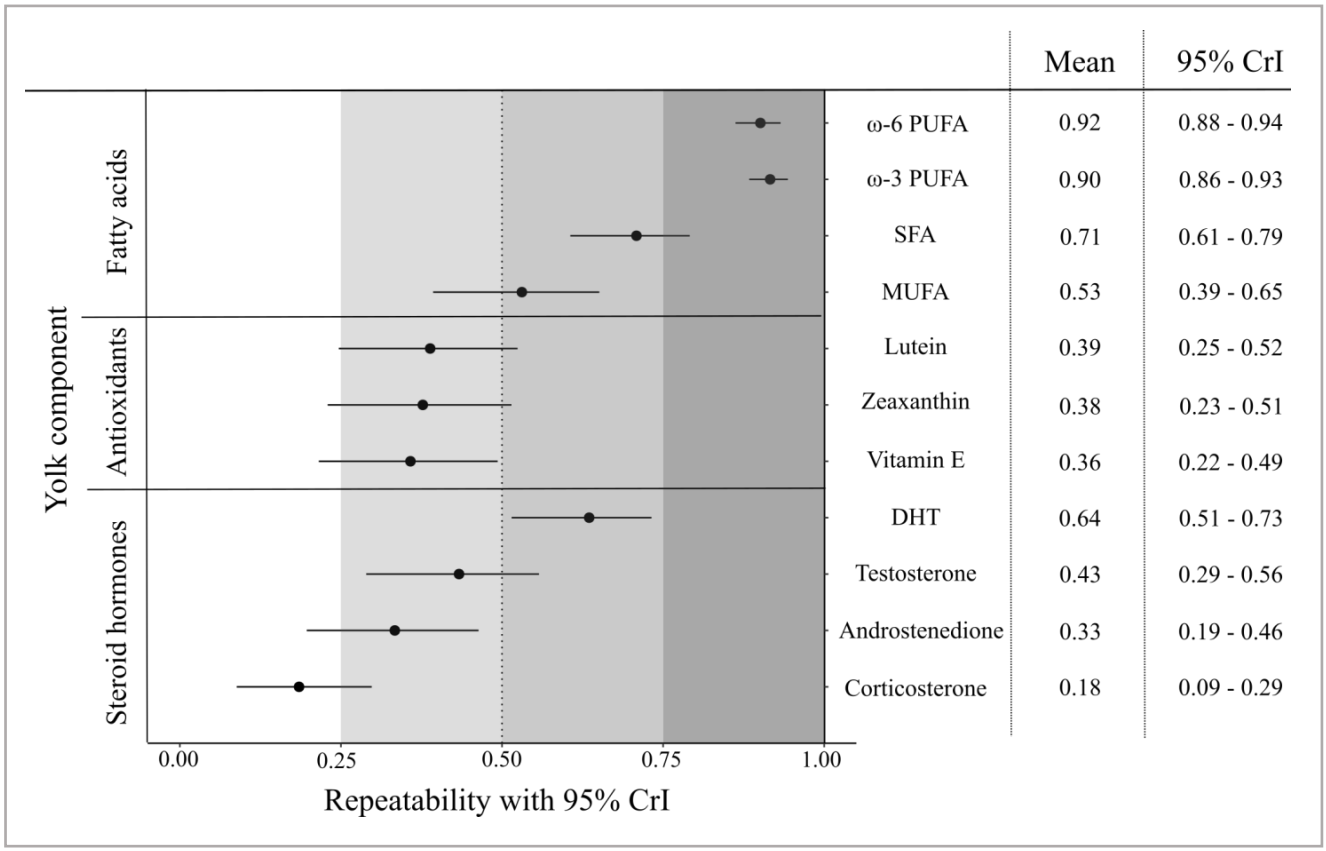

Figure 3.4. Adjusted repeatabilities of fatty acids (top), antioxidants (middle), and steroid hormones (bottom) of yolk components among females. Repeatability estimates (black circles in the graph and values in the left column of the table) and 95% credible intervals (CrI, horizontal lines in graph and numbers in right column of table) were obtained from linear mixed-effects models. Gray shading of increasing intensity indicates increases in repeatability. DHT, 5α-dihydrotestosterone; vitamin E, α-tocopherol; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA polyunsaturated fatty acids.

3.5. Discussion

We quantified 11 yolk components and analyzed their variation along the laying sequence by acknowledging the multi-level nature of female resource allocation. At a population level, the concentrations/proportions of five resources in the yolk varied along the laying sequence: first-laid eggs generally contained higher concentrations of 5α-dihydrotestosterone, antioxidants and proportions of ω-6 PUFA, and lower proportions of saturated fatty acids than last-laid eggs (Table 3.1; Figure 3.2). This result implies that in general the physiological environment is rather different for offspring developing in first *versus* last eggs. Individual females allocated yolk components over the course of laying in a pattern that was not necessarily the same as observed at the population level (Table 3.1; Figure 3.3). Females differed in their mean allocation of components (i.e., differences in the elevation of reaction norms) indicating that some females allocated on average higher amounts of components into their eggs than other females, while they also varied in their plastic response over the laying sequence (i.e., differences in the slope of reaction norms; Table 3.1; Figure 3.3). In addition, for some yolk components these two parameters were correlated. Finally, egg component allocation was repeatable, i.e., the concentration/proportion of most yolk components was more similar among eggs from the same female than among eggs from different females (Figure 3.4). Overall, these results show that at both population- and female-level, the physiological environment of the offspring will be different depending on the egg from which they develop. However, even if females are plastic in their allocation of components along the laying sequence, those eggs laid by the same mother are more similar to each other as compared to the eggs laid by a different mother.

3.5.1. Mean phenotypic plasticity in yolk components along the laying sequence: population level

For yolk hormones, consistent species-specific patterns of allocation along the laying sequence have been described for avian eggs (reviewed by Groothuis et al. 2005, Gil 2008). However, opposing patterns for the same hormone have also been described for different populations of the same species (reviewed by Groothuis et al. 2005, Gil 2008). For example, in free-living great tits an increase in androstenedione concentrations from first to last egg has been reported (Tschirren et al., 2004; Lessells et al., 2016), whereas in a study on great tits from selection lines the mean phenotypic plasticity showed opposing patterns depending on behavioral traits (Groothuis et al. 2008), and in our study androstenedione did not change. Further, a decrease in 5α-

dihydrotestosterone along the laying sequence was reported by Lessells et al. (2016) and our study, but not in the other two above-mentioned studies. Testosterone concentrations increased over the laying sequence in Tschirren et al. (2004) and Groothuis et al. (2008; only females from the 'bold' line), but no such trend was observed by Lessells et al. (2016) and the current study. Finally, while in a previous study yolk corticosterone increased (Lessells et al. 2016), we found no change along the laying sequence. This lack of agreement among different great tit populations could be explained, on the one hand, by female quality (e.g., body condition; Supplementary information 9.3, Table 4), environmental conditions (e.g., mean ambient temperature; Table 3.1), consistent individual differences between females ("personality"; Ruuskanen et al. 2018), and social factors, such as territory quality and male condition or personality (Remeš 2011, Ruuskanen et al. 2018), which all may influence female yolk allocation. In addition, opposing population trends could also be explained by different patterns of individual female plasticity (see below).

In the current study, all three antioxidants (i.e., vitamin E, lutein, and zeaxanthin) decreased along the laying sequence, confirming previous studies showing that last-laid eggs generally have lower concentrations of antioxidants in birds (Royle et al. 1999, 2003, Hõrak et al. 2002, Blount et al. 2002, Rubolini et al. 2011; but see Török et al. 2007). Animals cannot synthesize vitamin E and carotenoids *de novo*, therefore these antioxidants have to be obtained from the diet (Surai and Speake 2008) and can then be allocated into the egg yolk (from where the developing embryo will absorb them; reviewed by Yigit et al. 2014). These antioxidants may represent a limiting resource for the mother (Møller et al. 2000), and indeed, females in better body condition on average allocated higher concentrations of vitamin E into their eggs (Supplementary information 9.3, Table 4). However, lesser black-backed gull (*Larus fuscus*) females supplemented with a diet rich in carotenoids had higher carotenoid concentrations in plasma and yolk, but they also decreased yolk carotenoid concentrations over the laying sequence in a similar way as non-supplemented birds (Blount et al. 2002). Since the last-laid eggs in our study were not inferior to first-laid eggs in terms of egg mass (which increased over the laying sequence; Supplementary information 9.3, Table 1), these findings suggest that the observed decline in antioxidants cannot be attributed solely to female depletion in nutrients and other resources over the laying period.

Eggs were collected at a time when great tits were changing their diet from mainly feeding on seeds to predominantly feeding on invertebrates (caterpillars) which, compared to seeds, are particularly rich in saturated fatty acids, the ω-3 PUFA α-linolenic acid, and carotenoids (Isaksson and Andersson 2007, Andersson et al. 2015, Isaksson et al. 2015). In addition, a strong correlation between fatty acid levels of ingested food and fatty acids in yolk has previously been established (Lin et al. 1991, Hulbert and Abbott 2011, Twining et al. 2016). Thus, the changes in fatty acid composition along the laying sequence reported here (i.e., an increase and decrease in the proportion of saturated fatty acids and ω-6 PUFA, respectively) may be explained by an increase in caterpillar availability over the course of the breeding season, and a lower reliance on seeds, which are richer in ω-6 PUFAs. In line with this idea, lay date had a statistically positive effect on saturated fatty acids and ω-3 PUFA proportions (Table 3.1). However, other factors may also contribute to the observed patters given that the laying period in great tits can be quite short (mean ± SD: 8.45 ± 1.13 days in this study). For instance, in contrast to PUFAs, saturated and monounsaturated fatty acids can be biosynthesized *de novo* by animals, and fatty acids can also be selectively mobilized from internal stores to plasma (Raclot 2003, Price et al. 2008), suggesting that female condition at the start of laying may also play a role for the fatty acid allocation. Lastly, to date only three studies have documented mean phenotypic plasticity in fatty acid proportions along the laying sequence in free-living birds (Bourgault et al. 2007; Toledo et al. 2016). In contrast to our findings, a recent study on several populations of great tits in the UK found no variation in fatty acid composition along the laying sequence (Toledo et al. 2016). In that study, however, fatty acid composition was analyzed only for the 2^{nd} to the 5^{th} eggs in the laying sequence, which is equivalent to analyzing yolks only from the first to the middle eggs in our study. After re-analyzing our data with only first to middle eggs (n = 49 eggs), the fatty acid proportion still changed along the laying sequence (results not shown), thus indicating that it is unlikely that the difference in the position of the eggs analyzed explains the differences in the patterns of allocation obtained in the two studies. On the other hand, Toledo et al. (2016) collected one egg per nest. Our finding that yolk fatty acid composition in general is more similar in eggs from the same mother compared to those from other mothers (i.e., high repeatability; see below) could explain the differences in allocation patterns reported in these two studies.

Finally, it is important to bear in mind that ethical considerations limited our sample size (i.e., of entire clutches collected), potentially reducing our statistical power to identify the environmental or internal factors underlying population-level variation in yolk steroid hormones, antioxidants and fatty acids allocation.

3.5.2. Co-secretion of yolk components

To date, most studies investigating the fitness consequences of yolk allocation patterns focused on single groups of yolk components. This approach has been important for understanding the ways in which females can generate transgenerational phenotypic plasticity in the offspring by allocating certain substances into their eggs, as well as the evolutionary consequences. However, such studies may misinterpret the fitness benefits of single yolk components because we now know that different classes of components are allocated at the same time, often targeting the same phenotypic traits in the offspring (e.g., Treidel et al. 2013). We simultaneously quantified yolk steroid hormones, antioxidants and fatty acids, components that are known to affect growth, immune responses and oxidative stress of offspring, but sometimes in opposite directions. Although our study cannot directly address patterns of co-secretion of certain components because of limitations in sample size (n=11 clutches), we have observed some tantalizing patterns of co-occurrence in our study population that merit further investigation. For instance, first-laid eggs were high in 5α-dihydrotestosterone concentrations and ω-6 PUFA proportions. Both of these components are essential to promote embryo development, but they can also potentially increase the concentration of reactive oxygen species and thereby induce oxidative stress (Pamplona et al. 2002, Larsson et al. 2004, Alonso-Alvarez et al. 2007, Hulbert and Abbott 2011). However, first-laid eggs also had high antioxidant concentrations, which can buffer oxidative stress (e.g., Royle et al. 2001, Surai et al. 2001, Watson et al. 2018). These findings raise the question of whether selection promotes females to allocate eggs with a particular yolk composition, for example by co-secreting substances that have growth-enhancing but oxidative-stress inducing effects (like androgens and PUFAs) together with substances that can mitigate oxidative damage like antioxidants. Supporting this idea, a positive correlation between testosterone and vitamin E concentrations in the yolk of 75 bird species was recently reported (Giraudeau and Ducatez 2016). Detailed studies of the co-secretion of several yolk components are now required to address the question of whether natural selection operates on female allocation patterns to balance the costs and benefits of allocating substances to the offspring. Furthermore, in addition to quantifying the fitness costs and benefits incurred by

females during the egg-laying stage (i.e., through resource acquisition, synthesis, allocation, etc.), future studies should also determine the costs and benefits that arise later at the offspring stage – to the mother (e.g., by having to provision more demanding offspring), to the offspring (e.g., by having a suboptimal phenotype given the environmental and social circumstances), and to the female's partner (e.g., by having to increase investment into parental care).

3.5.3. Variation in yolk components along the laying sequence: female level

Our findings provide the first evidence that females consistently differ in average amounts of yolk components *and* in their plasticity of allocation along the laying sequence (i.e., in their slope). This result could explain the existence of divergent mean phenotypic plasticity (i.e., at a population level) found in studies on different populations of the same species (reviewed by Groothuis et al. 2005, Gil 2008). Our current understanding of the mechanisms behind female variation in allocation patterns is limited. Female mean yolk androgen deposition shows moderate heritability (e.g., great tits, Ruuskanen et al. 2016). However, whether mean deposition of antioxidants and fatty acids is also partially explained by heritable variation still remains unknown. Furthermore, which genetic factors may underlie female phenotypic plasticity also represents an important unanswered question. Ecological parameters that affect female physiological condition like prevailing climatic conditions, female quality or population density could also potentially alter female yolk allocation along the laying sequence. Genetic and non-genetic sources can simultaneously affect both components of the reaction norm (i.e., the variation in the average amount of yolk components and female plasticity), thus contributing to the overall among-female variation observed.

The elevation-slope coefficients that we obtained in our analyses for yolk components like androstenedione, testosterone, carotenoids, vitamin E and MUFAs should be interpreted with caution because of the low sample sizes (Martin et al. 2011, van de Pol 2012). Nevertheless, our analyses allowed us to quantify the covariation between two sources of variation, i.e. the extent and the direction to which the elevation and slope in the allocation of one yolk component were correlated in females (Table 3.1; Figure 3.3). For example, a negative correlation between elevation and slope indicates that females that overall allocated a higher concentration of a component also decreased this component's concentration more strongly along the laying sequence (i.e., were more plastic). Such a pattern could suggest that females experienced a constraint along the laying

sequence. Furthermore, the presence of correlations between elevation and slope in yolk components indicates that consequences of such maternal effects cannot be fully evaluated by studying these two sources of variation independently. For those components where female mean allocation and individual plasticity are correlated, fitness consequences (e.g., number of chicks that hatched or fledged) that would be attributed to one source of variation, for example to mean yolk concentrations in testosterone through an analysis of only the elevation of the allocation reaction norm, might in fact be caused by the other component, i.e., the change in yolk testosterone concentrations along the laying sequence. Lastly, this finding also raises the question of whether females with different reaction norms experience divergent fitness consequences. In other words, do females that on average deposit a higher proportion of e.g., monounsaturated fatty acids but are less plastic along the laying sequence have higher reproductive success than females that deposit on average lower proportions of that components but are more plastic (Figure 3.3e)?

Female-level variation in yolk allocation as well as the basis behind such plasticity still is a largely unexplored field of research. Does selection shape the allocation of average levels of egg components, the degree of plasticity over the laying sequence, or the correlation between these two traits? Addressing these questions will require large sample sizes, which might be a limiting factor in this field of research for ethical reasons. However, combining data from different populations and research groups could be a rewarding avenue to overcome this obstacle and increase our knowledge of the evolutionary and ecological forces driving phenotypic female variation in yolk allocation.

3.5.4. Repeatability in yolk allocation

The repeatability estimates in the present study varied depending on type of yolk resources allocated (Figure 3.4). Ours is the first study to report (adjusted) repeatabilities for antioxidants and fatty acids in egg yolks. The medium-high repeatabilities observed for these two groups (ranging from 0.36 to 0.92, Figure 3.4) are perhaps not surprising since great tits usually lay one egg each day, and may have experienced homogeneous environmental conditions within this short time frame. In contrast, the lower repeatability reported here for yolk hormones might be due to the fact that steroid hormones are synthetized by the mother herself (Groothuis and Schwabl 2008, Gil 2008). Within the group of steroid hormones measured, corticosterone concentrations had the lowest repeatability estimates. Plasma corticosterone levels are known to fluctuate over short time

scales, and its concentration in yolk may be influenced by maternal circulating concentrations (Saino et al. 2005, Groothuis and Schwabl 2008, Pitk et al. 2012). Therefore, the low repeatability estimates obtained for corticosterone might result from variations in maternal plasma concentrations along the laying sequence. Interestingly, while in our study 5α-dihydrotestosterone was the steroid hormone with the highest repeatability estimate (R = 0.64), in another recent study on great tits this hormone showed the lowest repeatability value (R = 0.29; Lessells et al. 2016). However, care should be exercised when comparing different studies because repeatability is a coefficient between variance explained by female identity in relation to the total variance (Nakagawa and Schielzeth 2010). Differences among females in their ability to synthesize and/or allocate each yolk component, the methodology used to measure each component, or environmental conditions that might increase the residual (unmeasured) variance, can all modify repeatability estimates even when the among-female variance remains the same. We therefore propose that future studies should report both among-female variance and repeatability estimates to allow for a better comparison of female consistency in yolk allocation across populations.

Repeatability estimates are a useful tool for evolutionary ecologists because they enable the quantification of the upper limit to heritability (Boake 1989). These estimates therefore provide information about the potential genetic contribution to the measured phenotype as well as clues as to whether some traits might evolve in response to selection. In our study, repeatability estimates for antioxidants and fatty acids were high. However, this does not necessarily indicate a high heritability in the allocation of these components. High repeatability estimates in our study could also be explained by repeated measurements taken at very short intervals (Araya-Ajoy et al. 2015, Holtmann et al. 2017) and/or by the fact that we adjusted for environmental factors such as lay date and mean ambient temperature. Irrespective of differences in absolute estimates of repeatability, the finding that repeatabilities for almost all yolk components were moderate to high ($R \geq 0.30$, with the exception of corticosterone, Figure 3.4) indicates that the developmental environment for offspring of the same mother is largely similar. Importantly, the high repeatability estimates obtained also confirm that the method of analyzing a single egg (ideally the middle egg) from a nest is a suitable way to estimate clutch-level yolk composition in studies of wild populations (e.g., Giordano et al. 2014), a technique that allows to assess the consequences for offspring phenotypes and fitness.

3.6. Conclusions

The present study emphasizes the need to account for female variation in yolk allocation along the laying sequence at multiple levels as a way to increase our understanding on the evolutionary processes that shape female yolk allocation. At a population level, our study shows that the developmental environment provided by mothers is different for offspring developing in first *versus* last eggs for almost half of the 11 components measured. Although not analyzed quantitatively, our study raises the question whether the patterns of allocation observed for steroid hormones, antioxidants and fatty acids are the result of selection favoring a complementary allocation of yolk components. Interestingly, the patterns of allocation at an individual level differed from the general pattern observed at a population level. At a female level, individuals varied among each other in the average allocation of yolk components, in their plasticity along the laying sequence as well as in the correlation between both parameters. In addition, females were remarkably consistent in the allocation of the majority of yolk components, confirming that the method of collecting a single egg from a nest is a suitable way to estimate clutch-level yolk composition in studies of wild populations – at least in those that aim to quantify the consequences for offspring phenotypes. Future studies can now build on these findings and test these patterns and their consequences in other species. It would also be important to analyze whether individual females are consistent in their allocation of yolk components across clutches laid in the same or in different years and if female plasticity along the laying sequence varies across homogeneous *vs.* heterogeneous environments. Since female allocation may be key to understand patterns at a population level, using mixed models to study female allocation at multiple levels opens up promising fields of research.

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Authors' contributions

LM conceived the study, conducted the field work, analyzed the data and drafted the manuscript. LM and MH designed the study with input from WG and CI. LM and MT conducted egg and steroid analysis. CI and MNA supervised the antioxidant and fatty acid extractions and analyses. MH, WG, CI and MNA contributed to manuscript preparation. All authors approved of the final version of the manuscript.

Permits

All experimental procedures were conducted according to the legal requirements in Germany and were approved by the governmental authorities of Oberbayern, Germany (license number 55.2-1-54-2532-25-2015).

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Supporting information

The following Supporting Information is available for this article:

- Supplementary Information 9.3: tables.

Chapter 4

Why we should measure multiple yolk components: Yolk fatty acids, but not androgens, predict offspring fitness in wild birds

Lucia Mentesana, Martin N. Andersson, Stefania Casagrande, Wolfgang Goymann, Caroline Isaksson & Michaela Hau

4.1. Abstract

Phenotypic variation among individuals is the material selection acts on. Maternal effects are an important mechanism to increase phenotypic variation because mothers often influence the developmental environment their offspring experiences early in life. In birds, mothers concomitantly secret steroid hormones, antioxidants and fatty acids into their egg yolk; yolk components that are thought to influence the same phenotypic traits in offspring. Yet, researchers generally focus on the effect of single yolk components while little is known about their interactive effect on offspring phenotypes. Here, we measured 31 yolk components present in the eggs of a wild population of great tits (*Parus major*) studied over two breeding seasons. We ran a principal component analysis and studied the link between yolk components and fitness proxies, as well as morphological and physiological traits of offspring. Hatching and fledging success were primarily associated with yolk fatty acids, including saturated, mono- and polyunsaturated fatty acid groups. Androgens, antioxidants and fatty acids were related to markers for offspring oxidative status determined at two times during the nestling phase. Overall, our study provides the first evidence for a relationship between yolk fatty acids and offspring fitness proxies in a wild population. It also supports the idea that offspring phenotypes are the consequence of intricate interactions among yolk components that females deposit into their eggs. Hence, our findings suggest that maternal effects through yolk deposition should be addressed by determining the multivariate composition of the egg, and not by studying single yolk components separately.

4.2. Introduction

Individuals from one population differ in an array of traits like morphology, physiology and behavior. Natural selection acts on this phenotypic variation, shaping survival and reproductive prospects of individuals. Hence, understanding the causes of phenotypic variation is a major goal in evolutionary biology. Maternal effects have recently been shown to contribute substantially to phenotypic variation (Moore et al. 2019, Yin et al. 2019). Mothers shape their offspring phenotype not only through the genes they pass on to them, but also by influencing the environment their offspring experience during development (Bernardo 1996, Mousseau & Fox, 1998). However, the influence of mothers on the offspring phenotype can vary across traits (e.g., morphological or physiological), developmental stages of the offspring (e.g., early *vs* late development) and according to the environment, among others (Moore et al., 2019; Yin et al., 2019). These differences in the magnitude and direction of maternal effects suggest that we need to elucidate the complexity inherent in maternal effects. That is, to make progress in our understanding of how mothers act as sources of phenotypic variation, we need to quantify the strength of maternal effects at different developmental stages and environmental conditions.

In egg-laying species, mothers can influence the environment in which their embryos will develop by allocating different resources into the yolk, such as hormones (Groothuis et al., 2005; Gil, 2008; Groothuis & Schwabl, 2008), antioxidants (Surai 2000; Surai, 2002) or fatty acids (Noble & Cocchi, 1990; Surai & Speake, 2008), among many other components. The importance of some of these maternally transmitted compounds for phenotypic traits has been documented in various taxa (e.g., McCormick 1999; Royle et al. 1999; Lovern & Wade 2001; Saino et al. 2002; Dziminski et al. 2009). In birds, yolk steroid hormones such as androgens and glucocorticoids can promote offspring growth, competitive ability and survival, but they can also increase chick susceptibility to oxidative stress by increasing the production of reactive oxygen species or by impairing antioxidant defenses (Groothuis et al., 2005; Gil, 2008; Haussmann et al., 2012; Treidel et al., 2013). Maternally derived antioxidants, like carotenoids or vitamin E, can influence growth and limit the negative consequences of increased oxidative stress by scavenging the reactive oxygen species produced during growth (Surai, 2000; Surai, 2002; Surai 2012; Parolini et al., 2017; Watson et al., 2018). Fatty acids are an important source of energy; they can enhance the general viability and proper development of both embryos and nestlings (Noble & Cocchi 1990, Surai & Speake 2000). In particular, polyunsaturated fatty acids (PUFAs) are essential components for the

formation of cell membranes, heart functioning and brain development (e.g., Hulbert & Abbott 2011). At the same time, PUFAs can be damaged by lipid peroxidation through reactive oxygen species that are generated as a by-product of the embryonic metabolism (Pamplona et al. 2002, Larsson et al. 2004, Hulbert & Abbott 2011, Yigit et al., 2014, Watson et al. 2018). Thus far, however, the evidence supporting the relevance of fatty acids for embryo and nestling development and phenotype has come mainly from poultry or captive studies (reviewed by Twining et al., 2016a); the effects of yolk fatty acids on offspring phenotypes remain vastly unexplored in wild populations that experience natural fluctuations in food availability and weather (but see Toledo 2016).

Maternal effects through egg deposition is often studied by measuring the effect of single yolk components, in particular androgens, on offspring fitness and phenotypic traits (reviewed by Groothuis et al., 2019). This approach has been pivotal for advancing our knowledge of how mothers can affect offspring phenotypes. However, maternal effects are by nature multivariate. Mothers can simultaneously transfer different classes of yolk components. For example, maternally transmitted androgens and antioxidants are co-adjusted within eggs across bird species (Giraudeau & Ducatez 2016). Along similar lines, steroid hormones, antioxidants, and fatty acids can be co-secreted in eggs of wild great tits (*Parus major*; Supplementary information 9.4 & 9.6, Figure 1). In addition, as previously described, different yolk components are thought to influence the same offspring traits, potentially by interacting with each other. For instance, hatchling mass was reduced and oxidative stress increased in Japanese quail (*Coturnix japonica*) chicks hatching from eggs injected with either testosterone or carotenoids (Giraudeau et al., 2016). But when both components were administered together neither hatchling mass nor oxidative stress were affected. These studies therefore suggest that maternal substances work interactively within an egg, with the overall composition of yolk components jointly determining offspring phenotypes. It also indicates that the consequences of maternal effects on offspring phenotypes described in studies that focus on single yolk components might actually be absent, weakened, or potentiated if the presence and actions of other components are also quantified.

Here we studied the consequences of maternal yolk deposition for nestling fitness and phenotype in a wild population of great tits (*Parus major*) over two years. To test for the interactive effect of yolk components, we measured the concentrations of 31 yolk components in eggs from 69 clutches including four steroid hormones, three antioxidants and 24 fatty acids. We then analyzed the

relationships between these yolk components and fitness proxies as well as those between yolk components and phenotypic traits. In particular, we examined associations with nestling growth (i.e., mass and tarsus length) and oxidative status. The oxidative status of an individual is defined as the concentrations of pro-oxidants (i.e., reactive oxygen species) and antioxidants (i.e., non-enzymatic and enzymatic compounds) present in cells and tissues (Costantini 2019). A change in any of the molecular components of the oxidative system in favor of pro-oxidants can lead to damage of biomolecules such as lipids, proteins and DNA, with potential fitness consequences (Finkel & Holbrook, 2000). Because the strength of maternal effects can change with stage of development (i.e., soon after hatching nestlings might rely more on prenatal conditions than when ready to fledge) and newly hatched nestlings have not yet fully developed their antioxidant system (Surai 1999), we measured chick growth and oxidative status at two time points: during early development and during exponential growth (i.e., days 6 and 12 post-hatching, respectively). We also collected data on ambient temperature and rainfall during the entire breeding season since these are key environmental factors known to affect fitness in songbirds (Visser et al. 1998; Öberg et al., 2015).

4.3. Materials and methods

4.3.1. Study species and field site

The study was carried out in the Dellinger Buchet, in Southern Germany (Bavaria; 48˚03' N, 11˚13' E, 620 m above sea level) from April to July in 2015 and 2016. We studied a wild nest-box population of great tits (*Parus major* L., Passeriformes) in a forest with a mosaic of deciduous and coniferous patches. Female great tits generally lay one egg each day and the clutch size varies from 6 to 12 eggs (Haftorn, 1981). Incubation starts after the last egg was laid, and it lasts on average 15 days (mean ± SD = 14.07 ± 2.77). There is a low percentage of females that lay a second clutch (~ 18%). For the purpose of this study, only first clutches were considered in the analysis.

4.3.2. Nest monitoring and egg collection

Nests were visited every second day from the beginning of the breeding season onwards. Once egg laying started, nests were visited every day and eggs were marked with a pencil to identify the laying order. The fourth egg was collected on the date of lay between 8:00 and 13:00 h and it was replaced with a dummy egg. A previous study reported that yolk components showed medium-

high repeatabilities for androgens (R = 0.30 – 0.64) and antioxidants (R = 0.36 – 0.39), and high repeatabilities for fatty acids (R > 0.5 for SFAs and MUFAs and R > 0.9 for PUFA; Mentesana et al., 2019). These repeatability estimates indicate that the concentration of yolk components in eggs laid by the same female are more similar to each other than the concentration of yolk components in eggs laid by other females, thus validating the approach of collecting the middle egg of each clutch as a representation of average female yolk deposition patterns (for a similar approach see, for example, Tschirren et al., 2009; Giordano et al., 2014). In total, 69 eggs were collected. All collected eggs were laid by different females, except for two females that bred in both years. Once in the laboratory, freshly laid eggs were weighed, opened, and the yolk was separated from the albumen by rolling it on a piece of paper (following the protocol by Lessells et al., 2002). The yolk was then homogenized in distilled water and stored at -80°C until further analysis.

4.3.3. Nestling growth monitoring and blood sampling

Nests were checked until clutch completion, as identified by the absence of freshly laid eggs and females incubating their eggs. Two days prior to expected hatching, each nest was monitored daily to record the hatching date (day 0 = the day the first hatchlings were observed). On day 1, nestlings were individually identified by clipping several down feathers and body mass (to the nearest 0.1 g) was recorded. On day 6 or 7 (hereafter referred to as day 6 for simplicity), a small blood sample (~ 20 μl) was collected from the branchial vein with heparinized capillaries for determination of the oxidative status of each nestling. All nestlings (N = 182) from the same nests (N = 51) were sampled within 15 min (mean ± SD = 12.72 ± 6.93). Body mass and tarsus length (to the nearest 0.1 mm) were also recorded. On day 12 or 13 after hatching (here after referred to as day 12 for simplicity), another blood sample (~ 80 μl) was collected for analysis of oxidative state. Samples were taken within 3 min (mean ± SD = 1.86 ± 0.73) from two or three chicks (N = 96) randomly selected per nest (N = 36). Body mass, tarsus and wing lengths (to the nearest 0.1 mm) were also recorded. Chicks were then individually marked with a numbered aluminum ring. On day 15, final body mass, tarsus and wing lengths were recorded. Finally, fledging was monitored until all offspring left the nest.

4.3.4. Yolk analyses

In total, we measured 31 yolk components (Supplementary information 9.5, Table 1). Steroids hormones (androstenedione, 5α-dihydrotestosterone, testosterone, and corticosterone) were

separated via diatomaceous earth column chromatography following the method described by Wingfield and Farner (1976), modified by Goymann et al. (2008) with additional adjustments for the measurement of egg yolk following Schwabl (1993). For a detailed description of the method see Mentesana et al. (2019). Androgen concentrations were quantified using radioimmunoassays, and corticosterone concentrations were determined with enzyme immunoassays. Androstenedione, 5α-dihydrotestosterone, testosterone, and corticosterone concentrations were extracted from the yolk in three sets of assays. Recoveries for the these sets of columns for androgens were within the expected range previously reported for great tits (Tschirren et al., 2004; Groothuis et al., 2008; Lessells et al., 2016; Mentesana et al., 2019), and were as follows (mean ± SD): androstenedione = 80.33 ± 4.93%, 5α-dihydrotestosterone = 56.33 ± 4.04%, testosterone = 66 ± 15.62%. Duplicates of 100 µl were used for the radioimmunoassays. The hormone concentration of each sample was corrected for the individual extraction efficiency. Polyclonal antibodies used were the following: AN6-22 for androstenedione, DT3-351 for 5α-dihydrotestosterone and T3-125 for testosterone (all Esoterix Endocrinology, CA, USA). The lower detection limit was at 0.71 pg/ml for androstenedione, 0.66 pg/ml for 5α-dihydrotestosterone, and 0.39 pg/ml for testosterone. Blanks were all below detection limits. All samples were analyzed in two assays. The intra-assay coefficient of variation, as determined from the positive controls containing stripped chicken plasma pools, were androstenedione = 9.6%, 5α-dihydrotestosterone = 14.9%, and testosterone = 23.8%. The inter-assay coefficient of variation, as determined by including the first positive control of each assay, were: androstenedione = 13.3%, 5α-dihydrotestosterone = 13.6%, and testosterone = 22.3%. Corticosterone concentrations were determined using enzyme immunoassays (Lot No: 12041402D & 04281702, Enzo Life Sciences, Germany). An aliquot of 80 µl was used to estimate individual extraction recoveries (mean ± SD recoveries were 55 ± 5.29%). Duplicates of 100 µl were added to individual wells and samples were distributed across five assays. The intra-assay coefficients of variation were 0.69, 0.90, 1.49, 0.85, and 0.80%, and the inter-assay variation was 1.35%. Antioxidants (lutein, zeaxanthin and vitamin E) were extracted and then quantified by high performance liquid chromatography (HPLC) following Mentesana et al. (2019). Antioxidants concentrations were calculated from standard curves made from lutein, zeaxanthin and tocopherol, along with corrections for their respective internal standards. Of the 69 eggs analyzed, data on the concentration of lutein and zeaxanthin were missing for one and two eggs, respectively. We assigned the average population concentration value of each antioxidant to those eggs with missing values (Nakagawa & Ereckleton 2008). Fatty acids (saturated fatty acids: SFA; monounsaturated

fatty acids: MUFA; ω-3 and ω-6 polyunsaturated fatty acids: PUFA) were extracted and subsequently analyzed using gas chromatography-mass spectrometry (GC-MS) according to previously established methods (Eikenaar et al. 2017, Mentesana et al. 2019). Fatty acids concentrations were calculated by correcting the area of the curve obtained for each fatty acid by the respective area of the internal standard.

4.3.5. Oxidative biomarkers measurements in nestlings

On day 6, the concentrations of non-enzymatic antioxidants and reactive oxygen species were measured in nestling plasma. Antioxidant concentrations (expressed as mM HOCl neutralized) were measured using the OXY-Adsorbent test (Diacron International SRL, Grosseto, Italy) following the protocol described by Costantini et al. (2006). Organic hydroperoxides (expressed as mM H2O2 equivalents) were measured using the d-ROMs test kit (Diacron International SRL, Grosseto, Italy) following the protocol described by Costantini et al. (2006). On day 12, in addition to the two parameters determined on day 6, we also analyzed the concentrations of the enzymatic antioxidant glutathione peroxidase (GPX; expressed as U/ml) in red blood cells using the Ransel assay (Randox Laboratories, Germany) following Costantini et al. (2011).

4.3.6. Environmental conditions

Ambient temperature (°C) was recorded every hour by 12 i-buttons (DS9093A+ Thermochron iButton) placed in different locations in the forest, while information on total rainfall (mm) was obtained from a meteorological station less than 5 km from the field site (Oberpfaffenhofen; 48° 05’ N, 11 ° 16' E, 583 m above sea level).

4.3.7. Statistical analysis

To test for the interactive effect of yolk components, we ran a Principal Component Analysis (PCA-correlation matrix) including all 31 yolk components measured. PCA was selected over other multivariate analyses because it is a data reduction method that can be used to characterize the correlation structure of a set of related variables (Budaev 2010, Paliy & Shankar, 2016). For each principal component we considered the variables that had absolutes value of squared loadings of higher than 0.2. Because there seems not to be a universal agreement as to which should be the minimum cutoff, we decided on that value based on the correlation between yolk components (Supplementary information 9.6, Figure 1) and our research question. Principal components one

(PC1), two (PC2), and three (PC3) accounted for 58% of the cumulative variance in our data (Supplementary information 9.5, Table 1). The mean values of the principal components differed between years (Supplementary information 9.5, Table 2). To account for year differences, we standardized the loadings since we could not include 'year' as a random (we only have two levels) nor as a fixed factor (limited sample size). That is, for each nest we first subtracted and then divided the loading of each principal component by the mean value of that principal component in the corresponding year. This allowed us to assess the importance of each yolk component irrespective of differences in mean values between years (Gelman & Hill, 2007).

To study the relationship between the yolk components in the fourth egg of clutches and fitness proxies for these clutches we first ran three generalized linear models, fitting clutch size, hatchling and fledgling number as response variables. PC1, PC2, PC3, and date (i.e., when the egg was collected) were included as covariates. In the models for hatchling and fledgling number, clutch size was also included as a covariate. One clutch was depredated during the incubation period and it was therefore excluded from the models for hatchling and fledgling number. For the model using fledgling number as a response variable, we excluded another clutch after inspection of the residuals (i.e., Cook's distance plot; see below). Female ID explained no variance when included as a random factor, and so it was excluded from all final models. Body weight at fledging is a key predictor of post-fledging survival in great tits (Tinbergen & Boerlijst, 1990). Thus, as another fitness proxy we also assessed the relationship between yolk components and nestling mass and tarsus length on day 15. These morphological variables were studied in separate models because yolk components can influence tarsus length (i.e., structural body size) independently of mass (e.g., Twining et al., 2016b). Because of sample size constrains, to reduce the number of explanatory variables we fitted the residuals of a linear regression between these two estimates of body condition and clutch size as response variables in two separate linear mixed effect models. PC1, PC2, PC3 and date were included as covariates in the model, and nest ID was fitted as a random factor. Environmental variables such as mean ambient temperature and mean rainfall during incubation were included in the model for hatchling number, while mean ambient temperature and mean rainfall during the nestling period were included in those models for fledgling number and body condition. Time of capture was also initially included in the models for nestling body condition. However, since neither of the environmental parameters nor time of capture explained

the response variables (results not shown), we excluded them from the final models to avoid overparameterization.

To study the relationship between egg components and the physiological condition of individual nestlings from a given brood we ran linear mixed effect models. We fitted OXY, GPX (only for day 12), ROMs, nestling mass and tarsus length (corrected for brood size), and growth rate ([mass on day 12 or 6 – mass on day 1]/[day 12 or 6 – day 1]) as response variables. We included PC1, PC2, PC3 and date of capture as covariates, and nest ID as a random factor. We initially included other covariates in the model based on their biological relevance to the study question. These were clutch size, time of capture, ambient temperature at time of capture and total sampling time (i.e., time from arrival at the nest until the end of blood sampling for each individual). But because of sample size constraints only variables that had an effect on the response variables were retained in the final model. In particular, clutch size was retained as a covariate in models analyzing OXY and growth rate, and on day 12 total sampling time was fitted as a covariate in the model for ROM levels.

All statistical analyses were performed in R statistical freeware R-3.3.3 (R Core Team 2013). Principal Component Analysis were performed using the 'prcomp' package. Statistical models, on the other hand, were performed using the 'lme4' and 'arm' packages in a Bayesian framework with non-informative priors. We assumed a Poisson error distribution for the generalized linear models and a Gaussian error distribution for the linear mixed effect models. In all cases, the residuals were checked visually for the model fit. Whenever necessary, response variables were transformed (details on transformations are provided in Supplementary information 9.5). We mean-centered all covariates (i.e., mean value = 0, standard deviation = 1) because covariates differed in their scales of magnitude. Model structure was based on the study question and the biology of the species rather than model selection. We subsequently used the 'sim' function to simulate values from the posterior distributions of model parameters. From 10000 simulations, we extracted the 95% Bayesian credible interval (CrI) around the mean (Gelman & Hill, 2007) and assessed statistical support by obtaining the posterior distribution of each parameter. CrI provide more valuable information than p-values, like for example, the uncertainty around the estimates. We use the term 'statistically meaningful' when the estimated effect differed from zero with a posterior probability higher than 0.95. The threshold of 5% would be equivalent to the significance level in a frequentist framework (for further details on statistical inference see Korner-Nievergelt et al., 2015).

4.4. Results

4.4.1. Integration of yolk components

Using PCA we identified the relationships among all 31 yolk components (Supplementary information 9.5, Table 1). PC1 was negatively associated with vitamin E, one type of MUFA (i.e., 20:1n-9) and all ω-6 PUFAs (i.e., 18:3n-6, 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6). PC2 was positively associated with four SFAs (i.e., 15:0, 16:0, 17:0 and 18:0), all but one MUFAs (i.e., 18:1n-9, 16:1n-9, 16:1n-7, 18:1n-7) and all ω-3 PUFAs (i.e., 18:3n-3, 20:5n-3, 22:5n-3, 22:6n-3). Androgens (i.e., androstenedione, 5α-dihydrotestosterone and testosterone), carotenoids (i.e., lutein, zeaxanthin), and two MUFAs (i.e., 18:1n-9, 16:1n-9) loaded positively onto PC3. To simplify the description of the results, hereafter all PCs are discussed within positive loadings (i.e., we transformed PC1 into positive values). Also, since almost all MUFAs were included in PC2 while only one or two were present in PC1 and PC3, the effect of MUFAs on nestling phenotype are discussed primarily with regard to the results obtained for PC2. Corticosterone was the only yolk component with a low loading in all PCs. In our population, the deposition of corticosterone into egg yolks is the least repeatable trait in female great tits ($R \sim 0.18$; Mentesana et al. 2019), thus concentrations measured in the fourth egg are not necessarily predicting its concentrations in the other eggs of the same clutch. Therefore, we decided to not analyze it any further in this study.

4.4.2. Egg components and fitness proxies

Nests with eggs that had higher SFA, MUFA and ω-3 PUFA concentrations had higher hatchling and fledgling numbers (Figure 4.1; Supplementary information 9.5, Table 3) than nests with eggs containing lower concentrations of these fatty acids. On the contrary, fledging success was lower in nests with eggs with increased concentrations of vitamin E and ω-6 PUFAs. Neither carotenoids nor androgens explained fitness proxies. Clutch size, fledgling mass and tarsus length were not related to any yolk component.

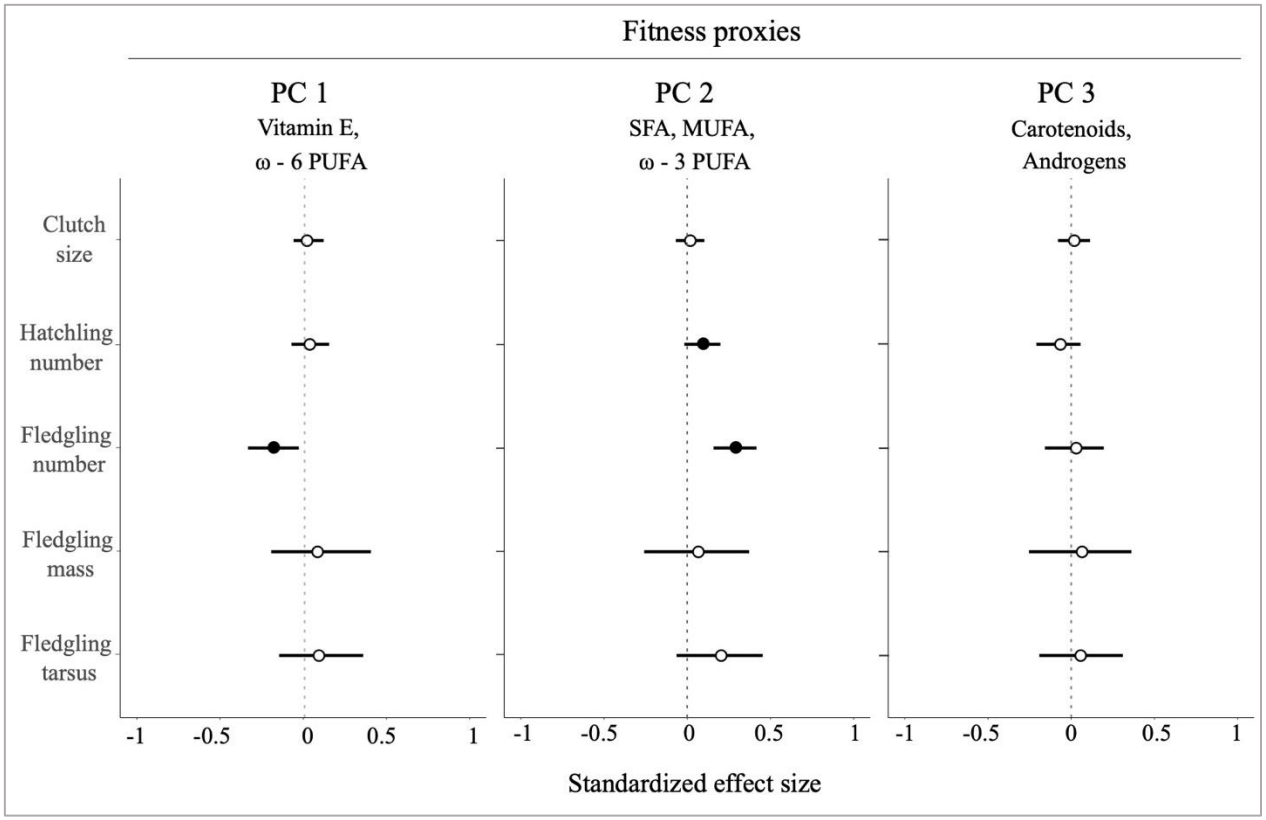

Figure 4.1: Relationships between egg yolk components and fitness proxies in great tit nestlings. Yolk components are grouped based on a Principal Component Analysis. PC1 was mainly represented by low concentrations of vitamin E (α - tocopherol) and ω-6 polyunsaturated fatty acids (PUFAs), PC2 by high concentrations of saturated (SFA), monounsaturated (MUFA) and all ω-3 PUFAs, and PC3 by high concentrations of androgens (androstenedione, 5α-dihydrotestosterone and testosterone) and carotenoids (lutein and zeaxanthin; see Supplementary information 9.5, Table 1 for a detailed description of the main yolk components included in each PC). Shown are the standardized effect sizes with their corresponding 95% credible intervals (CrI). Filled circles indicate statistically meaningful support (i.e., if the mean difference between compared estimates is higher than 0.95) for an effect of a set of yolk components on fitness proxies.

4.4.2. Egg components and phenotypic traits

On day 6, nestlings that hatched from eggs containing high concentrations of SFAs, MUFAs and ω-3 PUFAs had longer tarsi than nestlings hatching from eggs with low concentrations of these fatty acids (Figure 4.2a; Supplementary information 9.5, Table 4). Yolk carotenoid and androgen concentrations were negatively related to nestling OXY concentrations, while mass and growth rate were not associated with any group of components. On day 12, the oxidative status of great tit nestlings was explained by all yolk component groups, but in different ways (Figure 4.2b; Supplementary information 9.5, Table 5). In particular, nestlings hatching from eggs with high concentrations of vitamin E and ω-6 PUFA had higher levels of ROMs in plasma and lower levels of GPX in red blood cells. Plasma OXY concentrations were negatively related to high concentrations of SFAs, MUFAs, ω-3 PUFAs, and like on day 6, also with high concentrations of carotenoids and androgens.

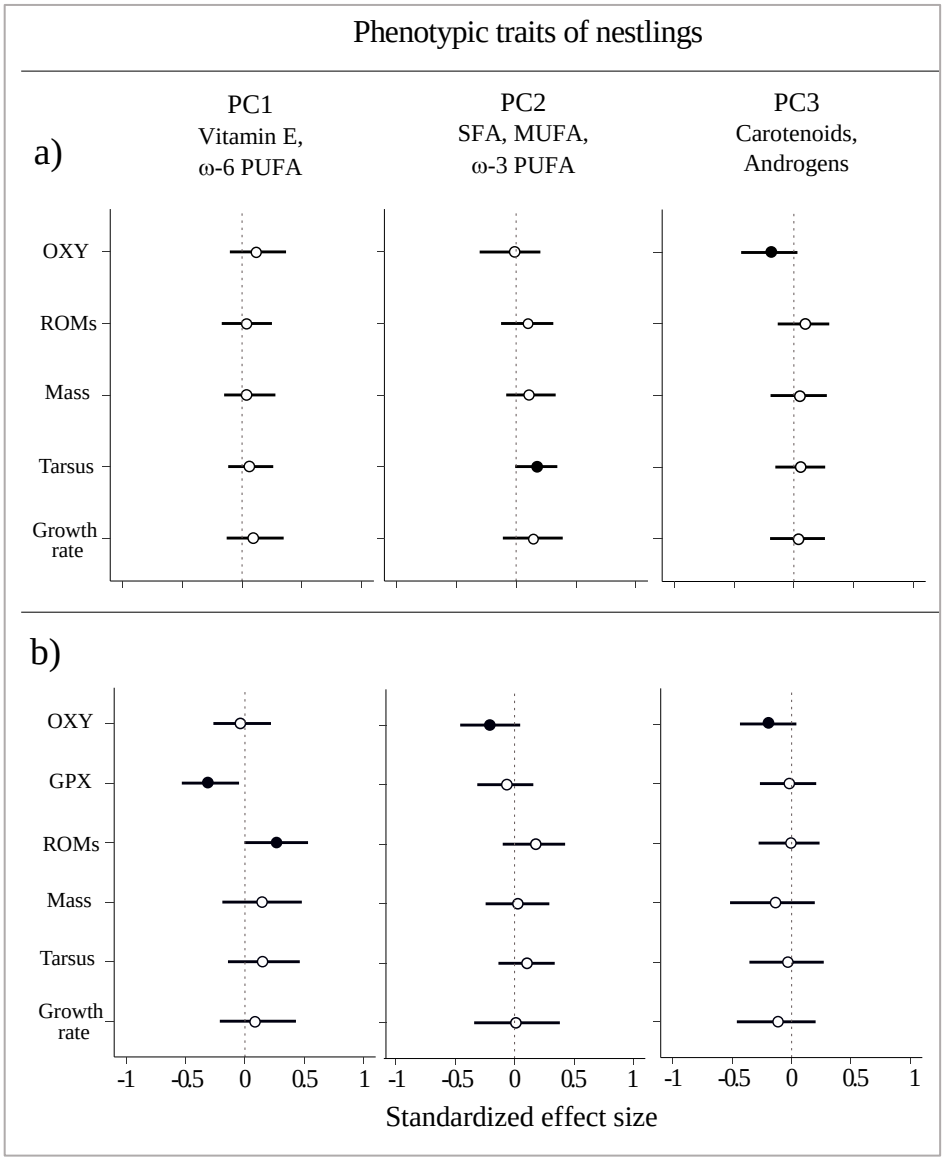

Figure 4.2: Relationships between egg yolk components and physiological traits in nestling great tits on days a) 6 and b) 12. Yolk components are grouped based on a Principal Component Analysis. PC1 was mainly represented by low concentrations of vitamin E (α - tocopherol) and ω-6 polyunsaturated fatty acids (PUFAs), PC2 by high concentrations of saturated (SFA), monounsaturated (MUFA) and all ω-3 PUFAs, and PC3 by high concentrations of androgens (androstenedione, 5α-dihydrotestosterone and testosterone) and carotenoids (lutein and zeaxanthin; see Supplementary information 9.5, Table 1 for a detailed description of the main yolk components included in each PC). Shown are the standardized effect sizes with their corresponding 95% credible intervals (CrI). Filled circles indicate statistically meaningful support (i.e., if the mean difference between compared estimates is higher than 0.95) for an effect of a set of yolk components on physiological traits. Oxidative status parameters: OXY, non-enzymatic antioxidants present in plasma; GPX, enzymatic antioxidant present in red blood cells; ROMs, reactive oxygen metabolites present in plasma. Growth rate was calculated as the change in mass from day a) 6 or b) 12 to 1 divided by the difference in the number of days.

4.5. Discussion

Fatty acids were the only group of yolk components that explained variation in nestling fitness-related traits, but not androgens and carotenoids – yolk components that were previously shown to affect chick development and fitness (e.g., Groothuis et al., 2005; Gil, 2008; Surai 2012). All component groupings explained aspects of the physiological phenotype of the nestlings (measures of oxidative status), albeit via different mechanisms. Together, our observations support the view that different yolk components in the egg interact with each other to shape the offspring phenotype.

5.1. Egg components and fitness proxies

Yolk fatty acids were related to hatching and fledging success. A relevance of fatty acids for fitness has been reported in marine and freshwater organisms (Brett & Müller-Navarra, 1997; Chen et al., 2012), but so far, this is the only study showing relationships between yolk fatty acid concentrations and fitness-related traits in a wild population. Hatching success was higher in nests of great tit mothers that laid eggs with an overall high concentration of saturated (i.e., SFA), monounsaturated (i.e., MUFA), and ω-3 polyunsaturated (i.e., PUFA) fatty acids (Figure 4.1). All fatty acids can be used as a source of energy, and when catabolized, they provide similar amounts of energy per mass (Hulbert & Abbott, 2011). However, SFAs and MUFAs are the ones generally used as energy sources because they can be endogenously produced (i.e., they are not essential fatty acids; Hulbert & Abbott 2011), are more abundant in the yolk (Toledo et al. 2016, Mentesana et al. 2019) and are less prone to oxidation (e.g., Pamplona et al. 2002). ω-3 PUFAs are particularly important in early life stages when organisms are developing and growing fast; they can enhance bone formation and immune competence (Watkins, 1991; Toledo 2016; Twining et al., 2016b). Thus, eggs containing high concentrations of SFAs, MUFAs and ω-3 PUFAs provide the embryo with both energy sources and essential components that allow for a proper development, and thereby may have boosted the hatching success of the chicks in this study.

Fledging success was also positively related to higher concentrations of SFAs, MUFAs and ω-3 PUFAs, while high concentrations of ω-6 PUFAs and the antioxidant vitamin E showed a negative association with fledgling numbers (Figure 4.1). We can offer three non-mutually exclusive explanations for these two results. First, SFAs, MUFAs and ω-3 PUFAs might have provided the nestlings with resources that enhanced growth during the first days after hatching (explained above; see also Toledo 2016; Twining et al., 2016b). Indeed, nestlings coming from eggs containing those

fatty acids had longer tarsi on day 6 (Figure 4.2a; Supplementary information 9.5, Table 4), which in our population is a good proxy of survival during the nestling phase (see Supplementary information 9.5, Table 6). Conversely, since in great tits MUFAs are negatively related to ω-6 PUFAs and vitamin E (Pearson's *r* - value = -0.42 & -0.41, respectively; $p < 0.001$; Supplementary information 9.6, Figure 1), eggs with higher concentrations of ω-6 PUFAs and vitamin E may have been more limited in these critical resources, leading to decreased fledging success. Second, early mortality in songbird nestlings is strongly influenced by the presence of pathogens (Møller, 1997). The relative concentrations of yolk PUFAs might have affected the development of the immune system which in great tit nestlings takes place early in life (Tschirren et al., 2004; Tschirren & Richner, 2006). A high concentration of ω-3 relative to ω-6 PUFAs can support anti-inflammatory processes, while pro-inflammatory responses occur when the relationship between these two PUFAs is the opposite (Larsson et al., 2004; Arts & Kohler, 2009). In this study, we did not measure nestling immune function. However, we observed that in great tit egg yolks there is a trend for an inverse relationship between the two types ω-PUFAs: eggs containing high concentrations of ω-3 PUFAs contained low concentrations of ω-6 PUFAs and vice versa (Pearson's *r* - value = -0.23, p=0.06; Supplementary information 9.6, Figure 1), raising the possibility that differences in fledging success among nests could be explained by differences in the inflammatory response of the nestlings. Lastly, the relationship between yolk composition and fledging success might be explained by the incorporation of food sources of different quality by females during egg laying. During this period, great tits change their diet from feeding on seeds to predominantly feeding on caterpillars (Perrins, 1991; Visser et al., 1998). If caterpillars are not available yet (because of low ambient temperatures) or are scarce (because of low habitat quality) adult great tits rely on seeds or other invertebrates such as arachnids (Naef-Daenzer et al., 2000). This change in food sources allows parents to maintain food intake but at the expense of impoverished food quality, because caterpillars are rich in SFA, ω-3 PUFA and antioxidants while seeds and arachnids are rich in ω-6 PUFA and vitamin E (Surai, 1999; Isaksson & Andersson, 2007; Andersson et al., 2015; Isaksson et al., 2015). Females can selectively mobilize fatty acids that are not acquired through the diet like SFAs and MUFAs from internal stores to plasma and then into the yolk, but since the concentrations of PUFAs and antioxidants in the yolk are the result of maternal diet, the food the female ingested can have a direct effect on fledging (and hatching) success (Raclot, 2003; Price et al., 2008). However, if mothers differentially allocate yolk resources to the eggs because they breed in forest patches with different food resources, it is also possible that differences in habitat quality,

and not in yolk composition *per se*, influenced fledging success via the food delivered by the parents to their young.

Fitness traits were not affected by the concentrations of androgens and carotenoids in the yolk. This is perhaps surprising given the large body of work focusing on how exposure to steroid hormones in early life can affect growth and survival (positively or negatively; e.g., Groothuis et al. 2005; Gil, 2008; Groothuis & Schwabl, 2008). However, recent studies suggest that yolk androgens are correlated with other yolk components (Giraudeau & Ducatez 2016, Mentesana et al. 2019). In fact, in our study yolk androstenedione hormone concentrations were positively correlated with both vitamin E and ω-6 PUFA (i.e., components related to fledgling success) in great tit eggs (Pearson's *r* - value = 0.54; $p < 0.001$; Supplementary information 9.6, Figure 1). This opens up the possibility that the effect of yolk androgens on fitness traits previously reported in studies measuring single yolk components may be indirect; perhaps through a link with other components highly correlated with yolk hormones. In line with this hypothesis, we found that androstenedione was positively related to fledgling success when analyzed as a single predictor (i.e., without including other yolk components in the model; Supplementary information 9.5, Table 7). Altogether, this supports the idea that egg components act interactively and that yolk fatty acid content is a component of importance for nestling fitness. Our results also highlight the importance of simultaneously measuring several yolk components in studies addressing the role of maternal effects as sources of phenotypic variation. Carefully designed studies manipulating egg components both in isolation and simultaneously are now needed to disentangle the independent role of each component as well as their joint action.

5.2. Egg components and phenotypic traits

Our study provides strong support for the view that maternal effects are an important cause of phenotypic variation among individuals, and that the influence of mothers on the offspring phenotype vary across traits and developmental stages of the offspring. In particular, yolk substances had their greatest influence on physiological rather than morphological traits, and mainly during the exponential growth phase of nestlings compared to early development. Besides the link between yolk fatty acids and fitness traits, yolk fatty acid content also explained variation in both morphological and physiological traits of nestlings (Figures 4.2a & b). On day 6, nestlings had longer tarsi when hatching from nests with eggs containing high concentrations of SFAs,

MUFAs and ω-3 PUFAs (Supplementary information 9.5, Table 4); a result that supports previous findings on the importance of fatty acids as energy sources and, in particular of ω-3 PUFA, for bone formation (Watkins, 1991; Hulbert & Abbott, 2011; Toledo 2016).

Androgens, antioxidants and all fatty acids simultaneously influenced the oxidative status of nestlings; albeit through different markers of the redox system (Figures 4.2a & b). On day 12, eggs containing high concentrations of SFAs, MUFAs and ω-3 PUFAs or high concentrations of androgens and carotenoids resulted in chicks with lower plasma concentrations of non-enzymatic antioxidants (i.e., OXY, Figure 4.2b, Supplementary information 9.5, Table 5), while ω-6 PUFA and vitamin E were associated with higher plasma concentrations of pro-oxidants (i.e., ROMs) and decreased enzymatic antioxidants (i.e., GPX) in red blood cells. A similar relationship of androgens and carotenoids with OXY was also present on day 6.

PUFAs are the fatty acids most susceptible to oxidation because of the high amount of double bonds they contain, thus high concentrations of PUFAs are expected to correlate with high concentrations of pro-oxidants (Pamplona et al. 2002). In our study this relationship was present only in the principal component that included high concentrations of ω-6 PUFA and vitamin E. However, the lack of association between ω-3 PUFA and ROMs might be explained by this yolk component being grouped with other fatty acids (i.e., in the principal component 2) and not analyzed individually. In fact, ω-3 PUFAs are present in low concentrations in the egg yolk compared to the other fatty acid groups (Toledo et al. 2016, Mentesana et al. 2019), potentially explaining why the principal component that included SFAs, MUFAs and ω-3 PUFAs was related to non-enzymatic antioxidants and not pro-oxidants. On the other hand, while androgens are generally associated with an increase in oxidative stress because they can promote growth and/or competitive ability (which in turn increase metabolic rate; e.g., Treidel et al. 2013), carotenoids have been shown to protect the embryo from the effects of androgens on oxidative stress (e.g., Surai 2012; Giraudeau et al., 2016). In our study, higher yolk concentrations of androgens and carotenoids were not associated with an increased growth in chicks. However, decreased plasma OXY concentrations in these chicks could have been a consequence of normal nestling growth and an insufficient scavenging of radical oxygen species by carotenoids.

The consequences of maternal yolk deposition on nestling oxidative status differed depending on developmental stage (e.g., nestling age). This may be shaped by the temporal dynamics of the nestling's developing antioxidant system. Vitamin E, which is determined by maternal diet composition, is the main antioxidant defense compound during avian embryogenesis and early development (e.g., Surai 1999, Watson et al. 2018). It plays a major role in terminating the free radical reaction generated by lipid peroxidation because it is localized in the cell membranes (i.e., together with PUFAs) and it can be recycled and regenerated (i.e., its lifetime is longer than that of other antioxidants; Surai, 1999). During embryonic development, yolk vitamin E is transferred to different tissues, mainly to the liver, and once hatched vitamin E becomes the main antioxidant for the nestlings until they develop their own antioxidant system (Surai, 1999). According to studies on poultry, two weeks after hatching plasma concentrations of vitamin E decrease and antioxidant protection is then mainly provided by enzymatic antioxidants (e.g., GPX; Surai, 1999). If we assume that the antioxidant system in wild birds develops in a similar way as in poultry, then during early development (i.e., on day 6) great tit chicks could have been protected from oxidative stress through ω-6 PUFA oxidation by vitamin E, while later, when nestlings experienced exponential growth (i.e., day 12) and their metabolic rate was high, nestlings could have relied on their own antioxidant system. This could potentially explain why on day 12 chicks that hatched from eggs with increased concentrations of vitamin E and ω-6 PUFAs had higher ROMs (which in our study was positively affected by an increase in mass and growth rate; Supplementary information 9.4 & 9.5, Tables 8 & 9) and lower GPX levels.

Variation in oxidative status markers might not necessarily have phenotypic or fitness consequences (reviewed by Costantini 2019). Unfortunately, because the recruitment rate of juveniles in our population was low (~ 7%), we could not measure whether the oxidative status of the nestlings (either early or late in the development) had consequences for these individuals later in life. However, the oxidative status of nestlings has been proposed as a potential mediator of survival and health (e.g., Sebastiano et al. 2017); thus, suggesting that the relationship between yolk components and oxidative status found in our study could ultimately translate into fitness consequences for the offspring.

4.6. Conclusions

Our study advances the field of maternal effects by providing evidence for a relationship between yolk fatty acids and offspring fitness proxies in a wild population. It also shows that different yolk components, belonging to different groups of compounds, affect the same phenotypic and fitness traits in the nestlings. This, together with previous studies showing that yolk components are co-secreted, suggests that the effects of maternal yolk deposition should be studied by simultaneously quantifying concentrations of several egg components rather than by focusing on single yolk components. To understand the function of specific yolk components, experimental studies are needed. However, such studies should carefully manipulate several yolk components simultaneously, and in a way that avoids disrupting their delicate balance. On a broader scale, since trait heritability is a prerequisite for evolution to occur, it would also be important to understand the degree to which the interaction between yolk components has a genetic basis versus the variation explained by current environmental conditions. To date, the mechanisms underlying maternal mean yolk deposition are still poorly understood: while yolk androgen deposition shows moderate heritability (e.g., Tschirren et al. 2009; Okuliarova et al., 2011; Ruuskanen et al., 2016) it is yet unknown to which extent the yolk provisioning with antioxidants and fatty acids can be explained by heritable variation.

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Authors' contributions

LM and MH designed the study. LM conducted field work, analyzed the data and drafted the manuscript. LM conducted egg and steroid analysis with supervision from WG. LM extracted and analyzed egg antioxidant and fatty acid extraction with supervision from MNA and CI. LM and SC conducted the oxidative strate lab analysis. All authors contributed to manuscript preparation and approved the final version of the manuscript.

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Permits

All experimental procedures were conducted according to the legal requirements in Germany and were approved by the governmental authorities of Oberbayern, Germany.

Supporting information

The following Supporting Information is available for this article:

- Supplementary Information 9.4: materials and methods.
- Supplementary Information 9.5: tables.
- Supplementary Information 9.6: figure.

5. General discussion

In this thesis, I determined the fitness consequences of physiological responses to environmental variation in a free-living population of great tits. I did so by studying how the physiological response of parents to environmental conditions related to patterns of reproductive investment, reproductive success and offspring fitness proxies.

This section starts by summarizing my main research questions and findings, followed by an expanded discussion of the chapters with additional results and future directions not included in the discussion sections of the previous chapters.

5.1. Synthesis

Glucocorticoids, oxidative state markers and body condition respond to environmental signals and help maintain homeostasis in an organism through change. Because of this, these physiological traits have been used to predict individual fitness proxies like reproductive success. However, identifying general patterns has proven difficult in wild populations of vertebrates; potentially, because of variation in the physiological state of individuals and in their response to environmental fluctuations. In chapter 2, I tested the hypothesis that glucocorticoids, oxidative state markers and body condition covary, and that they predict fitness proxies primarily under challenging environmental conditions. I used data on six physiological traits and two fitness proxies collected over two breeding seasons (May-June 2015 & 2016) that significantly differed in the environmental conditions. I found that glucocorticoids (i.e., baseline and stress-induced), oxidative state markers (i.e., enzymatic- and non-enzymatic antioxidants and pro-oxidants), and body condition do not covary, but they predict, in a sex-specific way, reproductive success when environmental conditions are challenging. Chapter 2 suggests that glucocorticoids, oxidative state and body condition might be important mediators to successfully cope with challenging environmental circumstances. This chapter also highlights that measuring the environmental context and including this information into our research question is key to understand when and why we observe relationships between physiological variables and fitness proxies.

The way in which mothers cope with environmental changes can affect patterns of reproductive investment by altering the resources mothers pass on to their developing embryos, such as hormones, nutrients and immune components. In egg laying species, females deposit these

components into their eggs. In birds, the patterns of deposition along the laying sequence vary across and within species, but the processes that shape female yolk deposition are not yet fully understood. In chapter 3, I used a statistical approach based on repeated measures to study female yolk deposition along the laying sequence at both the population and among-female levels. For this, I used data on 11 groups of yolk components measured in the eggs of 11 entire clutches collected during one breeding season. I found that the patterns of deposition at the population level differ from the ones at the among-female level. At a population level, I found that the concentrations/proportions of five yolk components vary along the laying sequence, implying that the developmental environment is different for offspring developing in first versus consecutive eggs. At the among-female level, I found that females are remarkably consistent and plastic in the deposition of yolk components along the laying sequence, and that for some components these two traits covary. Chapter 3 shows that variation in yolk deposition at a population level is underpinned by different individual patterns. It also suggests that female deposition may be shaped by both genetic and environmental components, and that for some compounds, females might be constrained in how much of a component they deposit on average into the eggs and how plastic such deposition can be along the laying sequence.

The prenatal environment provided by the mothers to the developing embryos can affect several phenotypic traits in the offspring and have fitness consequences. Yet, whether maternal yolk components work interactively within an egg, with the overall composition of yolk components jointly determining offspring phenotypes, remains poorly understood. In chapter 4, I used data on 31 yolk components measured in the fourth egg from 69 nests collected over two breeding seasons, to test for interactive effects of yolk components and to investigate their relationships with fitness proxies and offspring phenotypic traits. I found a relationship between yolk fatty acids and offspring fitness proxies; yolk components that are strongly influenced by the quantity and quality of the food consumed by mothers during egg laying. I also found that offspring phenotypes are related to the interaction among yolk components that females provide to their eggs. Chapter 4 highlights the importance of quantifying the multivariate composition of the egg (i.e., not study single yolk components separately) to fully understand among female variation in yolk deposition.

5.2. When and why do we observe a relationship between physiological traits and fitness?

In wild populations of adult great tits, non-significant, positive and negative relationships between glucocorticoids, oxidative state parameters and fitness proxies (i.e., reproductive success) have been reported (Table 5.1). Such mixed results were found in studies of populations living in largely similar habitat and climatic conditions (in Southern Germany within < 250 km of each other; Ouyang et al. 2012 & 2013 – chapter 2; this thesis) as well as more divergent habitats and climates (in Portugal versus Germany, at least 1500 km apart; Norte et al. 2010, Ouyang et al. 2012 & 2013). Relationships also differ within populations between the sexes (e.g., Norte et al. 2010, Ouyang et al. 2012, chapter 2) and across years (e.g., Ouyang et al. 2012, chapter 2).

Physiological traits		Relationship with reproductive success		Differences across years	Article
		Females	Males		
Glucocorticoids	Baseline	+	+	No	Ouyang et al. (2012)
		-	-	No	Ouyang et al. (2013)
		x/x*	x/+*(1)	Yes	Chapter 2 – this thesis
	Stress-induced	x	-‡	Yes	Ouyang et al. (2012)
		x	-	No	Ouyang et al. (2013)
		x/x*	x/-*(1)	Yes	Chapter 2 – this thesis
Oxidative state	OXY	x/x*	x/x*	No	Chapter 2 – this thesis
	GPX	+	x	No	Norte et al. (2010)
		+(1)/x*	x/x*	Yes	Chapter 2 – this thesis
	ROMs	x/-*	x/x*	Yes	Chapter 2 – this thesis
Body condition		+	+	No	Norte et al. (2010)
		+	-	Yes	Ouyang et al. (2012)
		x	x	No	Ouyang et al. (2013)
		x/x*	+(1)/x*	Yes	Chapter 2 – this thesis

Table 5.1: Summary of the currently available studies in wild populations of adult great tits reporting non-significant (x), positive (+) and negative (-) relationships between physiological traits and reproductive success. Reproductive success refers to the number of fledglings, except for * which refers to the mass of the nestlings and ‡ which refers to brood abandonment (a negative sign indicates that individuals with high stress-induced glucocorticoid concentrations had lower reproductive success because they were more likely to abandon their nests). (1) indicates a statistically meaningful effect present only in one year. OXY, non-enzymatic antioxidant; GPX, enzymatic antioxidant, ROMs, pro-oxidant concentrations. Body condition refers to body mass relative to body size, except in the study by Ouyang et al. (2012) where body condition refers only to body mass. Highlighted in grey are the results of my thesis.

These mixed results are not specific to great tits. Studies done in other bird species, as well as in other vertebrates (i.e., fish, reptiles, and mammals) have also consistently highlighted the lack of general patterns in how glucocorticoids and oxidative state correlate with fitness traits (Breuner et al. 2008; Bonier et al. 2009; Crespi et al. 2013; Costantini 2014, Speakman & Garrat 2014; Speakman et al. 2015; Blount et al. 2016, Romero & Wingfield 2016, Costantini 2018; Schoenle et al. 2019).

One explanation for the discrepancy in the relationships between glucocorticoids, oxidative state and fitness reported for wild populations of vertebrates is that sources of variation, like environmental, ecological or sex-specific variation, are often not considered (highlighted also by e.g., Ouyang et al. 2015; Dantzer et al. 2016; Henderson et al. 2017; Marasco et al. 2017; Schoenle et al. 2018; Vitousek et al. 2018a; Grant et al. 2020). Fitness is expected to be promoted if the physiological state of an organism in response to challenging environmental conditions allows the organism to escape or mitigate the challenge. However, if the challenge cannot be escaped or mitigated or if organisms are experiencing mild environmental conditions a negative or no relationship with fitness is expected. In response to low ambient temperatures or food availability, the energy required for an individual to go about its normal routine increases. Body condition and baseline glucocorticoids are physiological traits linked with variation in energetic demands. In particular, baseline glucocorticoids can increase to modulate glucose availability, stimulate appetite, and increase foraging and locomotor activities (McEwen & Wingfield 2003; Romero et al. 2009; Landys et al. 2006; Romero & Wingfield 2016). Hence, individuals that can increase baseline glucocorticoid concentration or have a good body condition are likely to increase the ability to cope with harsh environmental conditions (e.g., low temperature or food abundance) by providing the individual with the energy sources needed. Supporting this idea, I found that only in the year with challenging environmental conditions males in better body condition and with higher baseline glucocorticoid concentrations had more and heavier nestlings than males with low baseline concentrations and poor body condition, respectively (Figure 2.3 & 2.4, chapter 2; see also Ouyang et al. 2015; Henderson et al. 2017; Vitousek et al. 2018). I found no relationships between traits in the year with mild conditions. Also, in the year with bad weather, males with high stress-induced concentrations had lighter nestlings; suggesting that an increase in glucocorticoids in response to an unexpected perturbation (e.g., capture-restrained protocol) surpassed the threshold at which the birds shut down their reproductive system (e.g., by delaying the time father return to the nest to

feed their chicks; McEwen & Wingfield 2003; Wingfield & Sapolsky 2003; Ouyang et al. 2012; Romero & Wingfield 2016). In my study, parents did not differ in the number of fledglings (Supplementary information 9.1, Table 1) nor in their provisioning rates between years (results not shown). This indicates that parents probably had to elevate their metabolic rate in the year with harsh environmental conditions to maintain reproduction. The enzymatic antioxidant GPX is directly involved in detoxifying the cells from pro-oxidants (e.g., Halliwell & Gutteridge 2007), and its increase is generally associated with an increase in metabolic rate (e.g., Norte et al. 2010; Casagrande & Hau 2018). In my study, females that raised more and heavier chicks had higher concentrations of GPX and lower concentrations of pro-oxidants, respectively (Figure 2.3 & 2.4, chapter 2). These results therefore suggest that reproductive success is promoted in those females that can increase GPX and buffer the production of pro-oxidants in response to challenging environmental conditions, potentially as an increase in their metabolic rate.

Overall, chapter 2 shows that adjustments in physiological traits to challenging external circumstances may be more relevant for fitness than traits expressed under mild conditions, and suggests that glucocorticoids, oxidative state and body condition might be important mediators to successfully cope with challenging environmental circumstances. This chapter also highlights that the relationship between physiological and fitness traits can only be understood in a framework that incorporates several sources of variation, such as the environment and both sexes (Box 5.1).

Box 5.1. Why the relationship between physiological state and fitness is sex-specific?

In wild populations of great tits, the relationships between glucocorticoids, oxidative state and body condition with reproductive success differs between males and females (Table 5.1). Sex-specific relationships of glucocorticoids, body condition and reproductive success under challenging environmental conditions have previously been described in other bird species (e.g., Wingfield et al. 1999; Ouyang et al. 2012; Vitousek et al. 2018a), and the findings of chapter 2 suggest that ecological conditions may also have a sex-specific effect on the relationship between oxidative status and reproductive success.

There are several non-exclusive factors that might explain these differences. In vertebrates, males and females can differ in many morphological and behavioral traits. For example, great tit males are more colorful, heavier and have longer tarsi than females; on the other hand, female great tits invest more in reproduction since they produce, incubate and brood eggs while males only contribute to feeding the chicks (chapter 3 & 4; Hinde 1952). Since the expression of many of these traits is linked to physiological mechanisms, it might be expected to find physiological differences between males and females (Hau 2007; Casagrande et al. 2016; Klein & Flanagan 2016; Vitousek et al. 2016; Costantini 2018; Vitousek et al. 2019). However, in my study I did not find differences in the circulating concentrations of physiological traits between sexes (except for the concentration of stress-induced glucocorticoids that were higher in females); thus, suggesting that differences in the relationship between physiological traits and reproductive success are not explained by their absolute concentrations (see also e.g., Ouyang et al. 2012). However, since I only measured glucocorticoids and oxidative state markers in the blood, differences in the concentration of these traits could occur in other tissues (e.g., Hau et al. 2016; Costantini 2019). Second, males and females might differ in their sensitivity to environmental conditions (Pogány et al. 2008; Ouyang et al. 2012; Ouyang et al. 2016; Vitousek et al. 2018a). Supporting this idea, in my study males appeared more responsive than females to local ambient temperatures and rainfall (i.e., more physiological traits were related to changes in short- and long-term weather conditions; Figure 2.4). Third, males and females might differ in transport mechanisms, in the number and distribution of receptors, or in the case of glucocorticoids, in the regulation of the HPA axis (e.g., the strength of negative feedback, Zavala et al. 2011). Lastly, it is important to note that I captured and measured great tits during two years in the field; thus, my findings reflect differences in male and female investment in current reproduction. Because fitness is determined by both reproduction and survival, it is likely the case that differences in current reproduction are related to differences between males and females in future reproduction or survival (e.g., van de Crommenacker et al. 2017).

Variation in the results reported across and within wild populations can also be explained by the study design, in which individuals are generally measured at a single time point. This approach hinges on the notion that a single measurement at one point in time represents the average value of a particular physiological trait. However, a limitation of this approach is that it works with unpartitioned variance (e.g., Dingemanse et al. 2010, Niemelä & Dingemanse 2018). That is, it does not provide information on the plasticity of individuals in response to environmental changes nor on the relationship between plasticity and average trait value (i.e., mean and slope components of a reaction norm model; e.g., Dingemanse et al. 2010). This is a critical point that might strongly affect the conclusions of a study, especially when studying physiological traits which are by nature plastic (see also Williams 2008; Hau & Goymann 2015; Hau et al. 2016; Taff & Vitousek 2016). First, fitness might be related to the capacity of individuals to change from one physiological state to the other when environmental conditions change. In fact, studies in wild populations have reported negligible to moderate repeatabilities for single time-point circulating glucocorticoids concentrations (i.e., baseline and stress-induced: 0 – 0.5; e.g., Baugh et al. 2014, Ouyang et al. 2011, Sparkman et al. 2014, Hau et al. 2016) and for oxidative state biomarkers (i.e., antioxidants and pro-oxidants: 0 – 0.65; e.g., Stier et al. 2012, van de Crommenacker et al. 2011, Herborn et al. 2016, Urvik et al. 2016, Récapet et al. 2019). This means that the residual (unexplained) variation accounts for the majority of the total variation in these traits, which can be >70% when repeatability estimates are low (e.g., Baugh et al. 2014). Although a high residual variation can be due to both environmental variation and measurement error, it can also be due to within-individual variance (e.g., Nakagawa & Schielzeth, 2010, Niemelä & Dingemanse 2018). Given that glucocorticoids and oxidative state markers are strongly affected by short- and long-term environmental conditions (chapter 2, Romero 2000, Lushchak 2011, Romero & Wingfield 2016, Marasco et al. 2017, Grant et al. 2020), it is possible that fitness also relates to the plasticity of individuals besides its average state. Likewise, the link between glucocorticoids, oxidative state and fitness might depend on the correlation between the average physiological condition of an individual and its plasticity. A positive relationship between both components of a reaction norm model would indicate that individuals that generally have, for example, high concentrations of baseline glucocorticoids (individual 1 - cross; Figure 5.1a) will also show a steeper increase in baseline concentrations (individual 1 – line; Figure 5.1a) in response to environmental fluctuations and therefore even higher concentrations of baseline when environmental conditions are harsh, compared to individuals that generally have low baseline concentrations (individual 3; Figure 5.1a). While

differences in mean baseline glucocorticoids concentrations might not be linked with differences in reproductive success under mild conditions among individuals, an increase in baseline concentrations in response to harsh weather conditions might be linked to higher reproductive success for those individuals that have an overall high baseline concentration (Figure 5.1b).

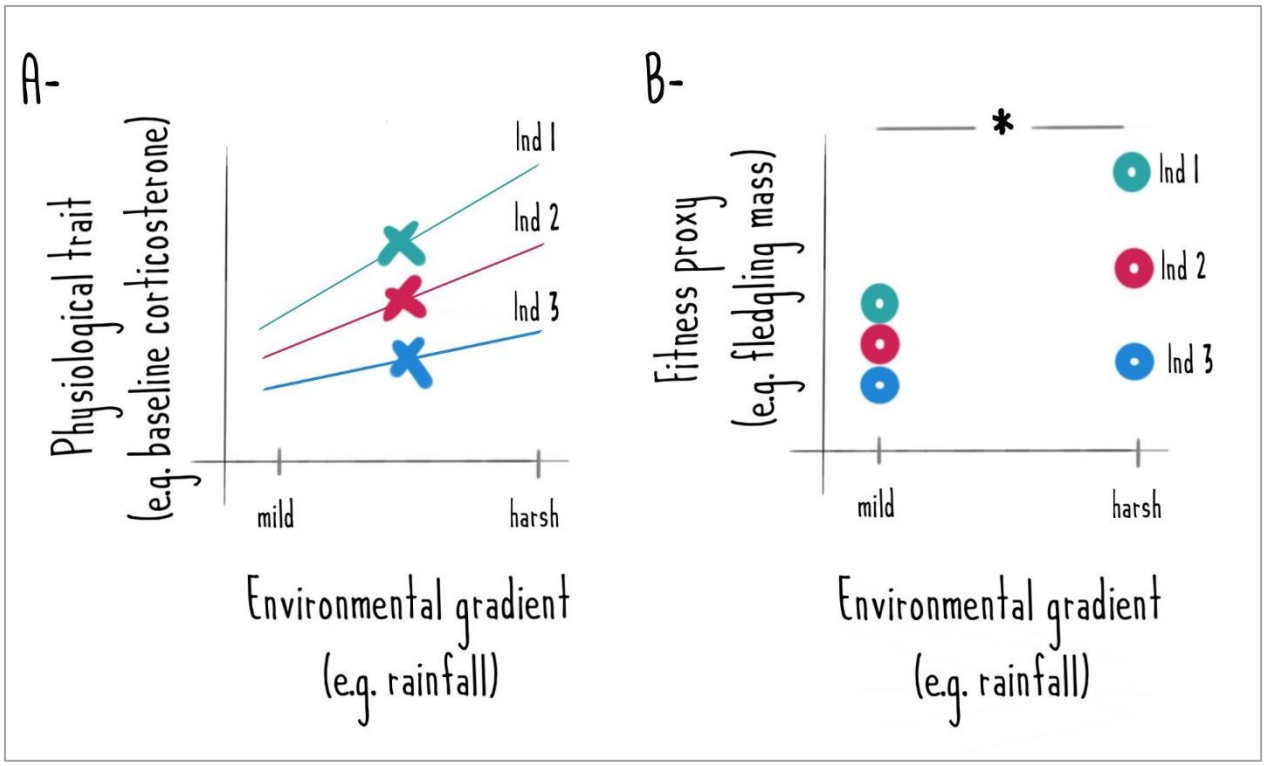

Figure 5.1. Schematic representation of **(a)** the relationship between a physiological state (e.g., baseline glucocorticoid concentrations) and environmental conditions (e.g., rainfall gradient) among-individuals that show a positive correlation between the mean value (indicated with a cross) and the plasticity of that physiological trait (indicated with a line). **(b)** Fitness consequences (e.g., fledgling mass) of individuals with different reaction norms at two different environmental contexts (e.g., low and high rainfall). * indicates significant differences.

There is growing evidence in the field of behavioral ecology that individuals show associations between consistent individual differences in the average level of a behavior across contexts (known as 'personality') and the extent to which individuals adjust this behavioral trait as a function of the environment (i.e., plasticity). For example, aggressive male golden hamsters (*Mesocricetus auratus*) do not adjust their level of bar pressing per reward as a function of experience, while less aggressive hamsters do (David et al. 2004), or active damselfish (*Pomacentrus bankanensis* & *Pomacentrus moluccensis)* have very similar activity rates across water temperatures while less

active damselfish have significantly higher activity rates when tested at higher temperatures (Biro et al. 2010). Given that personality-related differences in behavioral plasticity have been reported in all vertebrates (reviewed by Mathot et al. 2012, Mathot & Dingemanse 2014, Stamps & Biro 2016) and behaviors are generally mediated by physiological traits (e.g., hormones), it is likely that associations between the mean physiological state of an individual and its plasticity to respond to environmental stimuli may also exist. Importantly, a correlation between these traits would indicate constraints in the physiological capacity of individuals to meet environmental changes, potentially explaining why not all individuals can maximize their reproductive success under environmental fluctuations.

5.3. Do mothers show similar patterns of reproductive investment via yolk deposition?

The mother is the one that provides the first environment an individual encounters in its life. This prenatal environment is shaped by the resources that mothers pass on to their offspring; resources that largely depend on changes in the environment and the way females respond to these changes (Royle et al. 1999, 2003; Møller et al. 2000; Blount et al. 2002; Hõrak et al. 2002; Raclot 2003; Groothuis et al. 2005; Rubolini et al. 2011; Török et al. 2007; Surai & Speake 2008; Remeš 2011, Ruuskanen et al. 2018). In line with this idea, in chapter 3 current environmental conditions (e.g., mean ambient temperature, Supplementary information 9.3, Table 3.1), female body condition (body condition; Supplementary information 9.3, Table 4), and lay date (Supplementary information 9.3, Table 3.1) were related to female yolk deposition.

Females show different patterns of yolk deposition (chapter 3). This is perhaps surprising given that all eggs were collected within a month, during the same breeding season and in the same study area. But suggests that females within the same population and experiencing similar environmental conditions, differ in their investment in current reproduction (see also e.g., Groothuis et al. 2008). In particular, I found that these differences can be in the mean deposition of components, in their plasticity along the laying sequence and in the correlation between these two traits (chapter 3). Variation in the concentration of yolk components deposited by a female along the laying sequence indicates that the prenatal environment provided by mothers is different between first and last eggs. Remarkably, for some yolk components females consistently differ in average amounts of yolk components *and* in their plasticity of deposition along the laying sequence. For example, females that on average deposited a higher concentration of testosterone were less plastic along the laying

sequence than females that deposited on average lower concentrations of that hormone (Figure 3.3b), which suggest that females could experience a constraint in deposition along the laying sequence. Chapter 3 also shows that there are consistent among-female differences in yolk deposition along the laying sequence (i.e., female yolk deposition showed moderate to high repeatability; Figure 3.4). Repeatability estimates do not provide information on the mechanisms underlying among-individual differences (Wilson 2018), but there are two non-mutually exclusive explanations for these results. High repeatability estimates might be due to environmental characteristics (Araya- Ajoy et al. 2015, Holtmann et al. 2017, Niemelä & Dingemanse 2017, Wilson 2018) and/or by genetic differences among females (Boake 1989; but see Dohm 2002). In fact, female mean yolk androgen deposition in birds shows moderate heritability (Tschirren et al. 2009; Okuliarova et al. 2011; Ruuskanen et al. 2016). Hence, while the high repeatability estimates for antioxidant and essential fatty acid concentrations found in chapter 3 are mainly explained by current environmental factors (i.e., because these are components that can only be obtained by the mother through the diet), the deposition of steroid hormones, saturated and monounsaturated fatty acids might be shaped by both genetic and environmental components. Given that the concentrations of yolk fatty acids seem to be related to the reproductive success of mothers (chapter 4, see discussion below), understanding the mechanisms underlying the high consistency of female deposition of these components can provide insights into the evolutionary and ecological forces shaping maternal effects.

On the other hand, the information on repeatabilities confirms (for androgens and antioxidants) and extends (for fatty acids) data showing that analyzing a single egg from a nest is a suitable method to infer the overall condition of the clutch (see also Tschirren et al., 2009; Giordano et al., 2014; Tschirren et al. 2014). By using this approach, I found that androgens, antioxidants and fatty acids are co-secreted (Supplementary information 9.6, Figure 1). These findings raise the question of whether selection promotes females to allocate eggs with a particular yolk composition, for example by co-secreting substances that have growth-enhancing but oxidative-stress inducing effects (like androstenedione and ω-6 PUFA) together with substances that can mitigate oxidative damage like vitamin E. As a first step to understand this, I studied the consequences of the co-secretion of several yolk components for nestling fitness and phenotype (chapter 4).

5.4. Do mothers indirectly influence their reproductive success via their offspring?

The prenatal environment provided by the mothers to the developing embryos relates to offspring fitness traits. In chapter 4, hatchling and fledgling success were related to the concentrations of fatty acids and vitamin E (i.e., only for fledgling success) present in the egg yolk.

In birds, the concentrations of vitamin E and fatty acids in the yolk (and in the embryo) are strongly correlated with the concentrations of these components in the mother's plasma (Noble and Cocchi 1990; Lin et al. 1991, Surai 1999, Surai et al. 2008; Surai & Speake 2008). Remarkably, the majority of these components are essential; that is, mothers cannot produce them and need to acquire them through the diet (e.g., Surai 1999, Surai & Speake 2008, Hulbert and Abbott 2011; Twinning et al. 2016). Vitamin E and polyunsaturated fatty acids (i.e., ω-3 and ω-6 PUFAs) are essential components, while saturated and monounsaturated fatty acids can be both produced by the mother and obtained from food. Vitamin E (and tocopherols in general) are present in oil seeds, leaves, and other green parts of the plant (Surai 1999), while fatty acids can be obtained from seeds and plants but also from other animals (e.g., invertebrates and vertebrates, Hulbert & Abbot 2012, Andersson et al. 2015, Twinning et al. 2016). Variation in the diet of adults is reflected in the concentration of vitamin E and the proportion of fatty acids present in their tissues, such as blood (Hulbert & Abbot 2011, Andersson et al. 2015, Twinning et al. 2016). For instance, Andersson et al. (2015) measured the fatty acid composition of common food items of great tit like caterpillars, spiders and sunflower seeds. Andersson et al. (2015) found that sunflower seeds are rich in monounsaturated fatty acids and ω-6 PUFAs while insects are rich in saturated fatty acids and ω-3 PUFAs, and this variation in fatty acids composition also related to the variation observed in great tit plasma across seasons (e.g., winter vs summer) and habitats (e.g., urban vs rural). For my thesis, I collected eggs at a time when great tits change their diet from mainly feeding on seeds to predominantly feeding on invertebrates. Therefore, it is likely the case that the concentrations of vitamin E and fatty acids that I measured in the egg yolk of great tits were shaped by the quality and amount of food ingested by mothers during egg laying (e.g., Toledo et al. 2016, Parolini et al. 2019).

In Bavaria (Southern Germany), where my field site is located, events of beech (*Fagus sp.*) mast occur every three years (Waldbericht 2017). The seeds of beech trees are important food items for great tits, as well as for other birds, and events of beech mast are generally associated with higher annual survival (Perdeck et al. 2000). My thesis was conducted over two breeding seasons that had a high (2015) and low (2016) abundance of beech seeds (Waldbericht 2017). In these two years the proportion of yolk essential fatty acids in great tit eggs differed as well (Figure 5.2; unpublished data). In particular, the abundance of ω-6 PUFAs was significantly higher during the year of low abundance of beech seeds compared to the year with high abundance. Also, the composition of individual ω-6 PUFAs differed between years (e.g., pinolenic acid, 18:3n-6, was only present in the yolk the year with low abundance of beech seeds but not in the other year), thus suggesting different diets consumed by the mothers in the two years. In an attempt to understand if fluctuations in local conditions (i.e., mast events) may affect the concentrations of fatty acids in females, and therefore the presence of these components in their eggs, I measured the fatty acid composition of the seeds of the two main tree species present in my study site: spruce (*Picea sp.*), which is a coniferous evergreen tree in the family Pinaceae, and beech. These two species represent ~ 60% of the tree composition in my study site (28.31% and 28.08%, respectively; personal communication with the forester). For this, I first obtained seeds from the Botanical Garden in Munich (Germany) and then teamed up with researchers from Lund University (Sweden) to measure 19 fatty acids from the collected seeds. We found that the concentrations of fatty acids were different between seeds: seeds from spruce trees have only half of the proportion of ω-3 PUFA and almost the twice content of ω-6 PUFA than beech trees (Table 5.2, unpublished data), thus, suggesting that in years when mast events occur, great tits eat seeds that have a relative high abundance of ω-3 PUFA (i.e., yolk component positively related with hatchling and fledgling number) and low abundance of ω-6 PUFA (i.e., yolk component negatively related with fledgling number). This fatty acid composition found in seeds shows a similar pattern than the fatty acid composition we measured in the eggs over two years that differed in beech seeds abundance (Figure 5.2; unpublished data). Although these are exploratory analyses and we should be cautious in interpreting these results, fluctuations in seed abundance might shift female diet and therefore translate into variation in egg composition across years with potential fitness consequences.

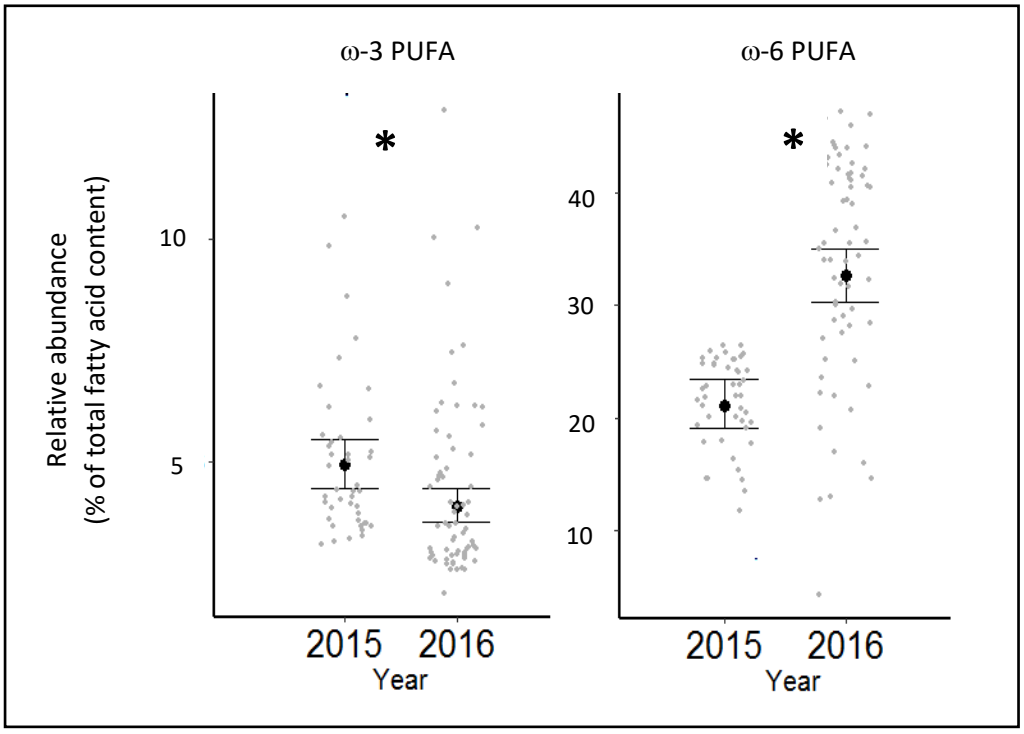

Figure 5.2: Relative abundance (% of total fatty acid content) of total ω-3 and ω-6 polyunsaturated fatty acids (PUFA) in great tit egg yolks. Grey circles show the individual data for egg yolks. Mean estimates of a linear model (black symbols) and 95% credible intervals (vertical bars) are also shown. * indicates significant differences.

Fatty acid	Food items	
	Spruce	Beech
ω-3 PUFA	2.5	4
ω-6 PUFA	80	47

Table 5.2: Relative abundance (% of total fatty acid content) of total ω-3 and ω-6 polyunsaturated fatty acids (PUFA) in seeds of spruce and beech trees. Events of beech mast are generally associated with higher annual survival in great tits (Perdeck et al. 2000). Fatty acid abundance was measured per gram of seed.

However, it is worth noting that my results do not allow me to address whether the relationship between yolk components and reproductive success is driven by single yolk components (and if so, if these are essential components or can be produced by the mother) or by the interactions among different components. Given the high correlation among yolk components (Supplementary Information 9.6, Figure 1; see also Postma et al. 2014; Tschirren et al. 2014; Giraudeau & Duckworth 2016) and the notion that several yolk components seem to influence similar phenotypic traits in the offspring (e.g., Giraudeau et al. 2016), in my thesis I tested the effect that multiple yolk components had on reproductive success (chapter 4). Testing which yolk components specifically relate to reproductive success could be done by using other types of statistical analysis (e.g., a full linear mixed model including all the yolk components measured or other multivariate analysis) than the one I used (i.e., Principal Component Analysis). But these analyses require a larger number of eggs than the ones I collected during my PhD (N = 69 eggs), which prevented me from running further post-hoc analyses. Future studies investigating if single components or specific interactions among yolk components relate to reproductive success may provide insights on potential constrains faced by females during deposition, thus shedding light on why there is variation in female deposition among females within and across populations (chapter 3, Groothuis et al. 2005, Gil 2008, Hsu et al. 2019). For example, if essential yolk components (i.e., vitamin E, ω-3 and/or ω-6 PUFAs) played a major role for female reproductive success and these components were not sufficiently available in the environment, females might be limited in the deposition of these components because of dietary limitations. In contrast, if the link between yolk components and reproductive success was driven by saturated or monounsaturated yolk components, which can be produced by the mother, variation in yolk deposition across females could be related to the physiological capacity of females to produce and mobilize these components from internal stores to plasma[1] (Raclot 2003, Price et al. 2008). Because there is evidence that the deposition of fatty acids and antioxidants observed in the eggs reflect a passive transfer from the mother's circulation (Noble and Cocchi 1990; Lin et al. 1991, Surai 1999, Surai et al. 2008; Surai & Speake 2008), the latter scenario would suggest that females face a physiological rather than dietary constraint in yolk deposition.

[1] Note that I recognize that this does not involve a conscious decision by the mother.

The way in which mothers interact with their environment also shapes the phenotype of the offspring (chapter 4). My results suggest that different yolk components simultaneously shape some of the same phenotypic traits. In particular, steroid hormones, antioxidants and fatty acids were linked to decreased concentrations of enzymatic and non-enzymatic antioxidants, as well as an increase in pro-oxidants; thus, indicating an overall perturbation in the oxidative state of the nestlings. There is considerable evidence that the oxidative state of individuals plays a central role in biological processes (Monaghan et al. 2009; Costantini et al. 2014; Blount et al. 2016; Vágási et al. 2019). Yet, given the complexity of the redox system (reviewed by Costantini et al. 2019) and the low recapture rate of juveniles in my population (~ 7%), I could not study whether such perturbations were translated into short- and long-term functional or fitness consequences for the offspring. However, if there are consequences for the offspring, my results suggest that these consequences will differ across offspring developing in eggs with different yolk composition. This is because, although yolk components simultaneously affected the redox condition of the nestling, different groups of yolk components were related to different markers of oxidative state; which can capture different aspects of the complex relationships between nestling oxidative state and reproduction or survival (Blount et al. 2006, Norte et al. 2008, Noguera et al. 2012, Christensen et al. 2016, Costantini 2019).

In my thesis I studied the consequences of average concentrations of yolk components (chapter 4). However, it might also well be that females differing in their degrees of plasticity along the laying sequence (chapter 3) produce eggs that differ in the balance of yolk components (and not necessarily the total concentration), with potential fitness or phenotypic consequences for the offspring. Furthermore, since for some yolk components both the average deposition of yolk components and the plasticity in deposition along the laying sequence were correlated (chapter 3), females with different covariance between these two traits might had divergent fitness or phenotypic consequences for the offspring. To test this, in a future step one could experimentally manipulate the yolk deposition of females (e.g., by feeding females different diets) such that females differ either in their mean deposition or in their plasticity of components, and measure the fitness consequences of such patterns of deposition.

The strength of maternal effects through yolk deposition can change with stage of development (e.g., Moore et al. 2019; Yin et al. 2019). Soon after hatching, nestlings might rely more on prenatal conditions than when ready to fledge. Hence, I cannot rule out the possibility that some relationships between yolk components and offspring phenotype or fitness traits (e.g., fledgling success) are also due to the postnatal conditions experienced by the offspring. Such conditions might be associated to direct environmental factors, such as ambient temperature, rainfall and or food availability (e.g., Visser et al. 1998; Öberg et al., 2015); although in my thesis neither mean ambient temperature nor mean rainfall during the nestling period were related to offspring fitness traits (chapter 4). Also, postnatal conditions can derive from the nutritional and physiological status of the parents or from further environmental changes generated by the parents. For instance, individual variation in both baseline and stress-induced glucocorticoid concentrations have been associated with variation in parental effort in a wide range of species (reviewed by e.g., Angelier & Chaster 2009; Bonier et al. 2009; Hau & Goymann 2015; Hau et al. 2016; Romero & Wingfield 2016). Further, the interaction between prenatal maternal effects and postnatal condition can also be an important determinant of offspring condition and fitness (e.g., Lindström 1999; Marshall & Uller 2007; Giordano et al. 2014). Yet, because some of the relationships reported in chapter 4 occur early in the development of the chicks (i.e., hatchling success and nestling growth soon after hatch; Figure 4.1 & 4.2), this chapter suggests that there are key maternal components, such as fatty acids, relevant for offspring phenotype and fitness traits.

5.5. Why should we take multiple measurements?

Measuring physiological traits repeatedly in individuals of wild populations is not easy (Fusani et al. 2005; Fusani 2008; Williams 2008; Hau & Goymann 2015; Hau et al. 2016). Direct measurements of physiological traits (e.g., glucocorticoids and oxidative state) typically require capturing, handling and taking blood samples of individuals. One problem often encountered in the field is that not all individuals can be recaptured. This is especially the case in migratory or open populations (i.e., where individuals are constantly on the move) and for short-lived species. In addition, handling individuals can sometimes interfere with the natural behavior of animals. Although this change in behavior seems to vary across contexts and species and in accordance with the internal condition of the individual (e.g., Ouyang et al. 2012; Schlicht & Kempenaers 2015, Goymann & Davila 2017), if a researcher is, for example, studying feeding behavior this might be a limiting point preventing him/her from taking multiple measurements. The study species and the

country where the study is carried out can also be limiting factors since projects aiming for multiple measuring might not be easily approved by animal care committees. Likewise, measuring several physiological traits simultaneously can also be difficult because it might require a bigger sample volume (e.g., blood or egg yolk, among others), more time, money and specific equipment to do all measurements. Also, the statistical analyses for repeated measures and/or multiple traits generally require big sample sizes (e.g., Martin et al. 2011, van de Pol 2012).

However, over the past years it has become more apparent that studies taking multiple measurements are a promising avenue of research that can bring about exciting findings (e.g., Boulton et al. 2015, Krams et al. 2017, Polverino et al. 2018). For example, there is a large body of work focusing on how exposure to steroid hormones in early life can affect growth and survival (positively or negatively; e.g., Schwabl et al. 1993; Groothuis et al. 2005; Gil, 2008; Groothuis & Schwabl, 2008). But recent studies suggest that the effect of these hormones on offspring traits are absent or weakened if the presence of other components (e.g., antioxidants or fatty acids) are also quantified (chapter 4; Giraudeau & Ducatez 2016; Parolini et al. 2019). Testing hypotheses by collecting multiple measurements for each individual can have several advantages over single-measurement studies: they can provide information on the correlation structures of physiological traits (chapter 2 & 4) and on variation in physiological traits at different hierarchical levels (e.g., among- and within-individuals; chapter 3). As discussed throughout this thesis, not controlling for these factors can contribute to our inability to generalize our findings on the relationships between physiological, phenotypic and fitness traits beyond our study species (see also e.g., van de Pol & Wright 2009; Haussmann & Marchetto 2010, Williams 2008; Cohen et al. 2012, Tschirren et al. 2014; Hau & Goymann 2015, Hau et al. 2016, Fowler et al. 2018, Niemelä & Dingemanse 2018). To overcome some of the difficulties associated with obtaining multiple measurements, researchers could collect samples over multiple years, have captive animals in semi-natural conditions, joint efforts with other research groups, and/or use non-invasive methods if they have been properly validated (see Goymann 2012 for the importance of proper biological and physiological validations of non-invasive methods).

5.6. Overall conclusions

Environments change continuously. Changes in an organism's environment can be challenging because they destabilize its homeostatic processes. Understanding if and how organisms can cope with environmental fluctuations has become critically important and urgently needed in the light of climate change and increasing human disturbance. My thesis shows that birds from a free-living population can successfully cope with unpredictable environmental disturbances during the breeding season, such as low temperatures and high cumulated rainfall, and suggests that glucocorticoids, oxidative state and body condition are important mediators to do so. However, my thesis also suggests that female reproductive success might be negatively affected via transgenerational effects on offspring fitness if fluctuations in environmental conditions cause a decrease in food supply, and therefore a decrease in essential yolk components transferred by the mothers into the eggs. Hence, studies measuring the interactions between parents and offspring fitness can provide new and important insights on our knowledge of how organisms cope with environmental changes.

6. References

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8. Author contributions

Chapter 2

Michaela Hau and I conceived and designed the study. I conducted the field work, run the corticosterone analysis in the lab, analyzed the data and drafted the manuscript. Stefania Casagrande and I conducted the oxidative stress lab analysis. Stefania Casagrande and Michaela Hau contributed to manuscript preparation.

Chapter 3

I conceived the study, conducted the field work, analyzed the data and drafted the manuscript. Michaela Hau and I jointly designed the study with input from Wolfgang Goymann and Caroline Isaksson. Monika Trappschuh and I conducted egg and steroid analysis. Caroline Isaksson and Martin N. Andersson supervised the antioxidant and fatty acid extractions and analyses. Michaela Hau, Wolfgang Goymann, Caroline Isaksson and MNA contributed to manuscript preparation.

Chapter 4

Michaela Hau and I jointly conceived and designed the study. I conducted field work, analyzed the data and wrote the manuscript. I conducted egg and steroid analysis with supervision from Wolfgang Goymann. I extracted and analyzed egg antioxidant and fatty acid extraction with supervision from Martin N. Andersson and Caroline Isaksson. Stefania Casagrande and I conducted the oxidative stress lab analysis. All authors were involved in revising the manuscript.

9. Supplementary information

9.1. Supplementary information chapter 2: tables

Table 1. Results from a linear model explaining inter-annual variation in environmental conditions (mean temperature and rainfall), fledgling mass and physiological traits (baseline and stress-induced corticosterone, OXY, GPX, ROMs, and body condition) during the breeding seasons of 2015-2016. The comparison of fledgling number between years was analyzed with a generalized linear model with a Poisson distribution. Statistically meaningful differences (i.e., if zero is not included within the 95% CrI) are given in bold font.

[a] OXY, non-enzymatic antioxidants in plasma.

[b] GPX, enzymatic antioxidant in red blood cells.

[c] ROMs, reactive oxygen metabolites in plasma.

[d] Scaled body mass index.

		2015 (Intercept)	2016
Environmental conditions	Temperature	13.51 (12.97; 14.05)	**-0.95 (-1.67; -0.24)**
	Rainfall	3.82 (3.49; 4.13)	**1.06 (0.65; 1.49)**
Reproductive success	Fledgling number	3.16 (1.90; 4.42)	-0.13 (-1.57; 1.32)
	Fledgling mass	16.52 (16.00; 17.03)	**-1.23 (-1.79; -0.66)**
Parental physiological parameters	Baseline corticosterone	4.79 (3.43; 6.14)	0.24 (-1.26; 1.75)
	Stress-induced corticosterone	33.17 (23.71; 42.35)	-1.49 (-11.69; 8.87)
	OXY [a]	281.13 (259.76; 302.12)	**-35.09 (-57.98; -11.52)**
	GPX [b]	16.79 (6.97; 26.36)	**14.20 (3.37; 25.08)**
	ROMs [c]	0.81 (0.53; 1.07)	0.07 (-0.22; 0.36)
	Body condition [d]	17.12 (16.74; 17.51)	0.00 (-0.43; 0.43)

Table 2. Results from generalized linear mixed-effects models with Poisson distribution and linear mixed-effect models estimating fixed and random effects to explain variation in (a) the number and (b) mass of fledglings in relation to maternal physiological traits. For model (b) we used the residuals of a linear regression between fledgling mass and brood size as the dependent variable. Each physiological variable was mean centered and fitted as a covariate, while year (2015 vs 2016) was fitted as a fixed factor. The interaction between each physiological variable and year was also included in the models. Band number and nest ID were fitted as random factors in the models fitting the number and mass of fledglings as dependent variables respectively. We present fixed (β) and random (σ2) parameters with their 95% credible intervals (CrI) in brackets.

[a] OXY, non-enzymatic antioxidants in plasma.

[b] GPX, enzymatic antioxidant in red blood cells.

[c] ROMs, reactive oxygen metabolites in plasma.

[d] Scaled body mass index.

(a) Fledgling number						(b) Fledgling mass (corrected for clutch size)					
Model 1		Model 2		Model 3		Model 1		Model 2		Model 3	
Fixed factors β (95% CrI)											
Intercept	0.66 (-0.07; 1.43)	Intercept	2.02 (0.63; 3.42)	Intercept	0.71 (0.07; 1.33)	Intercept	0.67 (-1.44; 2.81)	Intercept	2.12 (-4.25; 8.61)	Intercept	0.14 (-1.16; 1.45)
Baseline corticosterone	0.15 (-1.10; 1.40)	OXY [a]	-0.05 (-0.67; 0.58)	Body condition [d]	0.21 (-0.38; 0.81)	Baseline corticosterone	-0.13 (-5.29; 4.95)	OXY [a]	0.34 (-1.08; 1.78)	Body condition [d]	0.17 (-1.11; 1.44)
Stress-induced corticosterone	-0.06 (-1.48; 1.34)	GPX [b]	1.16 (-0.67; 3.01)	Year	0.18 (-0.47; 0.84)	Stress-induced corticosterone	0.79 (-5.13; 6.70)	GPX [b]	3.21 (-4.34; 10.93)	Year	-0.22 (-1.66; 1.21)
Year	0.48 (-0.31; 1.24)	ROMs [c]	1.24 (-0.48; 2.95)	Body condition * Year	-0.22 (-0.82; 0.39)	Year	-0.78 (-2.99; 1.42)	ROMs [c]	1.29 (-3.66; 6.36)	Body condition * Year	0.22 (-1.22; 1.64)
Baseline corticosterone * Year	-0.15 (-1.41; 1.13)	Year	-0.95 (-2.38; 0.46)	-	-	Baseline corticosterone * Year	0.29 (-4.85; 5.52)	Year	-2.14 (-8.70; 4.28)	-	-
Stress-induced corticosterone * Year	0.13 (-1.28; 1.58)	OXY * Year	-0.04 (-0.68; 0.61)	-	-	Stress-induced corticosterone * Year	-0.42 (-6.34; 5.56)	OXY * Year	-0.31 (-1.84; 1.24)	-	-
-		GPX * Year	-0.88 (-2.74; 0.94)	-	-	-	-	GPX * Year	-2.94 (-10.74; 4.58)	-	-
-		ROMs * Year	-1.21 (-2.91; -0.50)	-	-	-	-	ROMs * Year	-2.17 (-7.24; 2.74)	-	-
Random factors σ^2 (95% CrI)											
Band number	-	Band number	-	Band number	0.48 (0.33; 0.67)	Nest ID	2.20 (1.55; 3.06)	Nest ID	1.50 (1.04; 2.15)	Nest ID	2.06 (1.51; 2.75)
Residual variance	-	Residual variance	-	Residual variance	0.54 (0.39; 0.69)	Residual variance	1.69 (1.32; 2.17)	Residual variance	1.69 (1.33; 2.16)	Residual variance	1.66 (1.32; 2.10)

Table 3. Results from generalized linear mixed-effects models with Poisson distribution and linear mixed-effect models estimating fixed and random effects to explain variation in (a) the number and (b) mass of fledglings, respectively, in relation to paternal physiological traits. For model (b) we used the residuals of a linear regression between the mass of the nestlings and brood size as the dependent variable. Each physiological variable was mean centered and fitted as a covariate, while year (2015 vs 2016) was fitted as a fixed factor. The interaction between each physiological variable and year were also included in the models. Band number and nest ID were fitted as random factors in the models fitting the number and mass of fledglings as dependent variables respectively. We present fixed (β) and random (σ2) parameters with their 95% credible intervals (CrI) in brackets.

[a] OXY, non-enzymatic antioxidant present in plasma.

[b] GPX, enzymatic antioxidant present in red blood cells.

[c] ROMs, concentration of reactive oxygen metabolites present in plasma.

[d] Scaled body mass index.

a) Fledgling number						b) Fledgling mass (corrected for clutch size)					
Model 1		Model 2		Model 3		Model 1		Model 2		Model 3	
Fixed factors β (95% CrI)											
Intercept	1.09 (0.59; 1.58)	Intercept	0.98 (0.19; 1.76)	Intercept	1.15 (0.72; 1.57)	Intercept	0.63 (-0.40; -1.67)	Intercept	1.52 (-2.10; 5.10)	Intercept	0.74 (-0.29; 1.77)
Baseline corticosterone	-0.12 (-0.85; 0.60)	OXY [a]	-0.29 (-0.74; 0.16)	Body condition [d]	-0.39 (-0.91; 0.12)	Baseline corticosterone	0.43 (-1.30; 2.18)	OXY [a]	-0.18 (-1.78; 1.46)	Body condition [d]	-0.09 (-1.35; 1.11)
Stress-induced corticosterone	0.15 (-0.47; 0.77)	GPX [b]	-0.65 (-1.49; 0.20)	Year	-0.17 (-0.64; 0.31)	Stress-induced corticosterone	0.19 (-1.24; 1.59)	GPX [b]	0.81 (-3.07; 4.72)	Year	**-1.00 (-2.14; 0.17)**
Year	-0.02 (-0.14; 0.12)	ROMs [c]	0.15 (-0.26; 0.58)	Body condition * Year	0.60 (0.03; 1.18)	Year	-0.89 (-2.07; 0.27)	ROMs [c]	0.07 (-1.38; 1.54)	Body condition * Year	0.37 (-0.92; 1.74)
Baseline corticosterone * Year	0.15 (-0.60; 0.90)	Year	0.15 (-0.65; 0.95)	-	-	Baseline corticosterone * Year	0.10 (-1.76; 1.93)	Year	-1.84 (-5.51; 1.87)	-	
Stress-induced corticosterone * Year	-0.07 (-0.74; 0.61)	OXY * Sex	0.25 (-0.23; 0.75)	-	-	Stress-induced corticosterone * Year	-0.95 (-2.49; 0.62)	OXY * Year	0.11 (-1.72; 1.91)	-	
-	-	GPX * Year	**0.73 (-0.13; 1.59)**	-	-	-	-	GPX * Year	-0.47 (-4.51; 3.42)	-	
-	-	ROMs * Year	-0.12 (-0.59; 0.33)	-	-	-	-	ROMs * Year	-0.41 (-2.06; 1.24)	-	
Random factors σ^2 (95% CrI)											
Band number	0.24 (0.16; 0.35)	Band number	-	Band number	0.30 (0.20; 0.42)	Nest ID	1.65 (1.17; 2.23)	Nest ID	2.44 (1.67; 3.46)	Nest ID	1.88 (1.41; 2.45)
Residual variance	-	Residual variance	-	Residual variance	0.52 (0.39; 0.65)	Residual variance	1.61 (1.27; 2.02)	Residual variance	1.67 (1.29; 2.15)	Residual variance	1.53 (1.23; 1.89)

Table 4. Results from a generalized linear mixed-effects model with Poisson distribution and a linear mixed-effect model estimating fixed and random effects to explain variation in (a) the number and (b) mass of fledglings, respectively, in relation to mean weather conditions experienced by adults during the breeding season. For model (b) we used the residuals of a linear regression between the mass of the nestlings and brood size as the dependent variable. Mean ambient temperature and mean cumulative rainfall were mean centered and fitted as covariates, while year (2015 vs 2016) was fitted as a fixed factor. The interactions between each environmental factor and year were also included in the model. Nest ID was fitted as random factor. We present fixed (β) and random (σ2) parameters with their 95% credible intervals (CrI) in brackets.

(a) Fledgling number		(b) Fledgling mass (corrected for clutch size)	
Fixed factors β (95% CrI)			
Intercept	1.17 (0.27; 2.06)	Intercept	0.73 (-0.45; 1.91)
Mean temperature	0.53 (-0.98; 2.05)	Mean temperature	2.21 (-0.59; 4.94)
Mean cumulative rainfall	0.44 (-1.16; 2.02)	Mean cumulative rainfall	2.42 (-0.81; 5.60)
Year	-0.19 (-1.11; 0.73)	Year	-0.86 (-2.17; 0.44)
Mean temperature * Year	-0.61 (-2.14; 0.92)	Mean temperature * Year	-2.49 (-5.26; 0.37)
Mean cumulative rainfall * Year	-0.64 (-2.25; 0.98)	Mean cumulative rainfall * Year	-2.19 (-5.45; 1.15)
Random factors σ^2 (95% CrI)			
Band number	0.31 (0.21; 0.42)	Nest ID	2.07 (1.66; 2.59)
Residual variance	0.53 (0.34; 0.73)	Residual variance	1.41 (1.19; 1.64)

Table 5. Results from linear mixed-effects models estimating fixed and random effects to explain variation physiological components in relation to short- (i.e., at time of capture) and long-term (i.e., 72hs prior to capture) ambient temperature. Temperature and date were mean centered and fitted as covariates, while sex (females vs males) and year (2015 vs 2016) were fitted as a fixed factor. Two-way interactions between ambient temperature, sex and year were also included in the model. Band number and nest ID were fitted as random factors. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets.

[a] Baseline and stress-induced corticosterone values were square-root transformed.

[b] OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs reactive oxygen metabolites in plasma.

OXY, GPX and ROMs were square-root transformed.

[c] Scaled body mass index.

[d] Date of reproduction within a breeding season.

Short-term weather conditions							Long-term weather conditions						
	Corticosterone[a]		Oxidative status[b]			Body condition[c]		Corticosterone[a]		Oxidative status[b]			Body condition[c]
	Baseline	Stress-induced	OXY	GPX	ROMs			Baseline	Stress-induced	OXY	GPX	ROMs	
Fixed factors β (95% CrI)													
Intercept	2.12 (1.74; 2.50)	5.65 (4.69; 6.58)	16.60 (15.73; 17.49)	3.14 (1.97; 4.32)	0.81 (0.64; 0.99)	16.67 (16.22; 17.13)	Intercept	2.09 (1.72; 2.48)	5.56 (4.61; 6.50)	16.89 (15.88; 17.91)	3.25 (2.17; 4.33)	0.84 (0.66; 1.02)	16.63 (16.18; 17.10)
Ambient temperature	0.13 (-0.32; 0.56)	0.17 (-0.89; 1.26)	-0.05 (-1.04; 0.94)	0.67 (-0.69; 2.01)	0.13 (-0.08; 0.33)	-0.21 (-0.74; 0.32)	Mean ambient temperature	0.13 (-0.36; 0.61)	0.67 (-0.56; 1.86)	0.65 (-0.68; 1.94)	1.46 (0.15; 2.75)	0.03 (-0.19; 0.26)	0.06 (-0.54; 0.64)
Sex	-0.08 (-0.30; 0.15)	-0.03 (-0.73; 0.68)	0.22 (-0.16; 0.57)	0.20 (-0.66; 1.08)	0.01 (-0.09; 0.10)	0.51 (0.19; 0.82)	Sex	-0.09 (-0.32; 0.14)	-0.12 (-0.84; 0.60)	0.22 (-0.17; 0.61)	0.19 (-0.67; 1.07)	0.01 (-0.09; 0.11)	0.49 (0.18; 0.81)
Year	0.04 (-0.31; 0.39)	-0.39 (-1.25; 0.49)	-1.05 (-1.95; -0.15)	1.49 (0.49; 2.52)	0.05 (-0.13; 0.23)	0.19 (-0.23; 0.61)	Year	0.07 (-0.28; 0.42)	-0.26 (-1.16; 0.67)	-0.86 (-1.78; 0.04)	1.26 (0.31; 2.19)	0.01 (-0.16; 0.18)	0.23 (-0.35; 0.18)
Date[d]	-0.08 (-0.31; 0.14)	-0.14 (-0.67; 0.39)	-0.05 (-0.49; 0.38)	-0.56 (-1.23; 0.12)	-0.06 (-0.15; 0.03)	-0.11 (-0.38; 0.16)	Date[d]	-0.12 (-0.35; 0.11)	-0.20 (-0.77; 0.35)	-0.04 (-0.54; 0.35)	-0.74 (-1.37; -0.09)	-0.09 (-0.19; -0.01)	-0.17 (-0.45; 0.11)
Ambient temperature * Sex	-0.04 (-0.26; 0.19)	-0.21 (-0.91; 0.49)	-0.11 (-0.51; 0.28)	0.09 (-0.73; 0.93)	-0.00 (-0.10; 0.09)	0.13 (-0.18; 0.43)	Mean ambient temperature * Sex	0.04 (-0.17; 0.26)	-0.34 (-1.03; 0.36)	-0.11 (-0.49; 0.27)	0.04 (-0.71; 0.78)	0.02 (-0.07; 0.11)	0.05 (-0.23; 0.34)
Ambient temperature * Year	-0.22 (-0.65; 0.22)	-0.59 (-1.66; -0.48)	0.15 (-0.86; 1.15)	-0.91 (-2.23; 0.43)	-0.15 (-0.35; 0.06)	0.02 (-0.51; 0.55)	Mean ambient temperature * Year	-0.19 (-0.67; 0.31)	-0.75 (-1.94; 0.48)	-0.47 (-1.79; 0.88)	-1.44 (-2.77; -0.07)	0.02 (-0.21; 0.25)	-0.13 (-0.73; 0.48)
Random factors σ^2 (95% CrI)													
Band number	0.17 (0.12; 0.23)	1.14 (0.79; 1.58)	-	3.24 (2.42; 4.25)	0.01 (0.00; 0.01)	0.41 (0.29; 0.55)	Band number	0.24 (0.17; 0.32)	0.51 (0.35; 0.72)	-	4.84 (3.72; 6.22)	0.03 (0.02; 0.04)	0.39 (0.29; 0.54)
Nest ID	0.12 (0.07; 0.18)	0.02 (0.01; 0.03)	0.85 (0.62; 1.16)	0.35 (0.22; 0.53)	0.03 (0.02; 0.04)	0.13 (0.09; 0.19)	Nest ID	0.11 (0.07; 0.16)	-	0.83 (0.98; 1.12)	0.20 (0.12; 0.31)	0.02 (0.02; 0.04)	0.14 (0.09; 0.21)
Residual variance	0.12 (0.09; 0.16)	2.18 (1.64; 2.89)	0.70 (0.52; 0.96)	1.98 (1.50; 2.59)	0.04 (0.03; 0.06)	0.23 (0.17; 0.29)	Residual variance	0.07 (0.05; 0.09)	2.89 (2.18; 3.81)	0.72 (0.54; 0.98)	0.99 (0.76; 1.31)	0.02 (0.02; 0.03)	0.23 (0.18; 0.31)

Table 6. Results from linear mixed-effects models estimating fixed and random effects to explain variation physiological traits in relation to short- (i.e., at time of capture) and long-term (i.e., 72hs prior to capture) cumulative rainfall. Cumulative rainfall and date were mean centered and fitted as covariates, while sex (females vs males) and year (2015 vs 2016) were fitted as a fixed factor. Two-way interactions between rainfall, sex and year were also included in the model. Band number and nest ID were fitted as random factors. We present fixed (β) and random (σ2) parameters with their 95% credible intervals (CrI) in brackets.

[a] Baseline corticosterone values were log10 transformed and short- and long-term weather conditions were square-root transformed. Stress-induced corticosterone values were also square-root transformed.

[b] OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs reactive oxygen metabolites in plasma.

OXY, GPX and ROMs were square-root transformed.

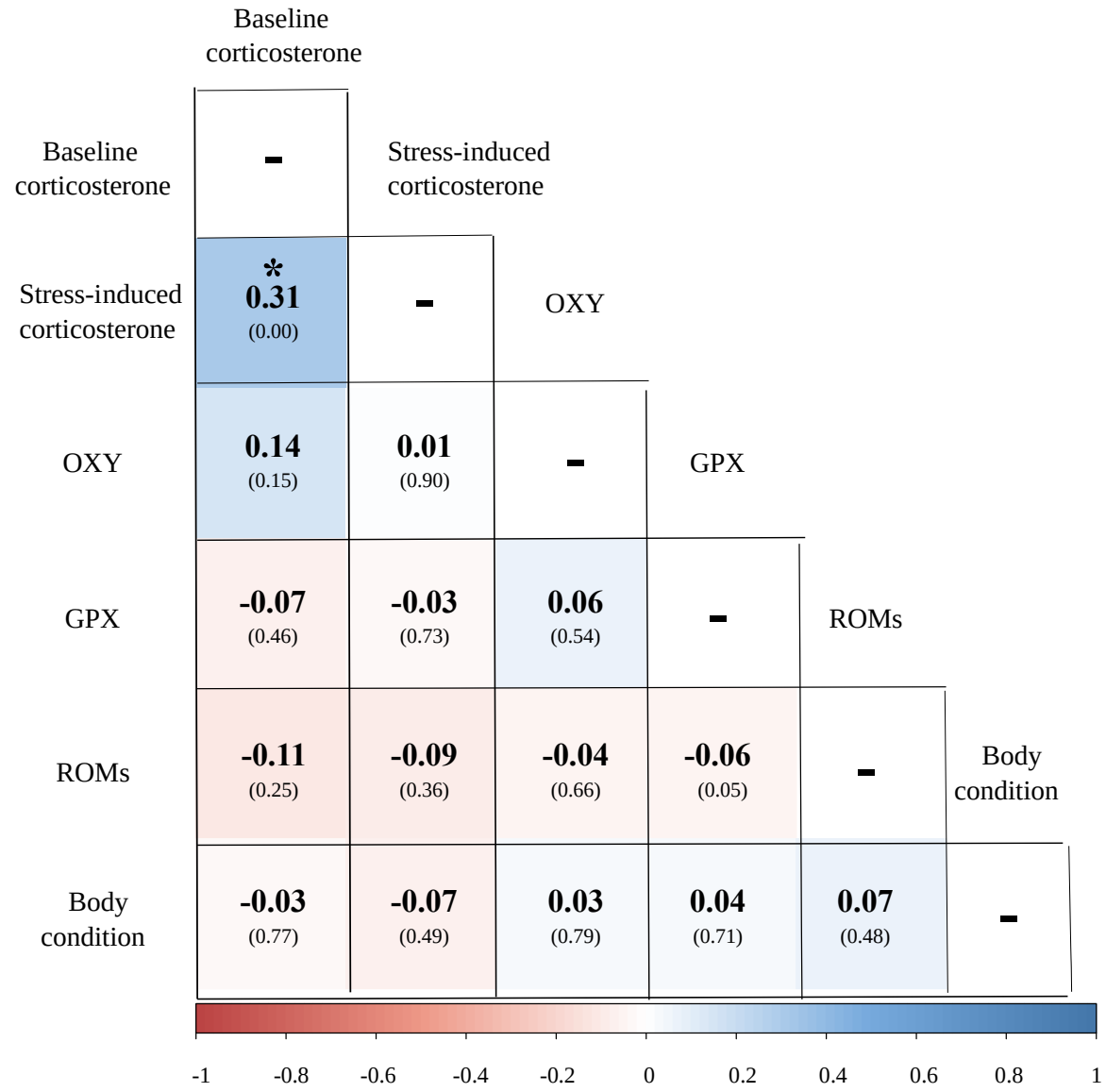
[c] Scaled body mass index.

[d] Date of reproduction within a breeding season.

Short-term weather conditions							Long-term weather conditions						
	Corticosterone[a]		Oxidative status[b]			Body condition[c]		Corticosterone[a]		Oxidative status[b]			Body condition[c]
	Baseline	Stress-induced	OXY	GPX	ROMs			Baseline	Stress-induced	OXY	GPX	ROMs	
Fixed factors β (95% CrI)													
Intercept	1.38 (1.02; 1.70)	5.23 (4.19; 6.26)	16.79 (15.86; 17.70)	3.09 (1.81; 4.38)	0.78 (0.61; 0.96)	16.74 (16.23; 17.25)	Intercept	2.10 (1.68; 2.52)	5.87 (4.65; 7.12)	16.84 (14.77; 18.50)	2.69 (1.46; 3.89)	0.96 (0.71; 1.21)	16.45 (15.91; 16.97)
Cumulative rainfall	-0.17 (-0.54; 0.21)	-0.58 (-1.79; 0.65)	0.74 (-0.40; 1.89)	0.18 (-1.44; 1.78)	-0.27 (-0.49; -0.04)	0.32 (-0.29; 0.91)	Mean cumulative rainfall	0.04 (-0.37; 0.45)	0.32 (-0.81; 1.46)	-0.05 (-1.76; 1.63)	-0.85 (-1.99; 0.27)	0.15 (-0.11; 0.40)	-0.14 (-0.65; 0.35)
Sex	-0.13 (-0.42; 0.13)	0.18 (-0.56; 0.93)	0.25 (-0.14; 0.65)	0.24 (-0.68; 1.15)	-0.00 (-0.10; 0.09)	0.53 (0.19; 0.85)	Sex	-0.09 (-0.32; 0.14)	-0.14 (-0.87; 0.58)	0.22 (-0.17; 0.59)	0.26 (-0.59; 1.13)	0.01 (-0.09; 0.10)	0.52 (0.21; 0.82)
Year	0.09 (-0.21; 0.39)	-0.15 (-1.15; 0.82)	-1.32 (-2.25; -0.38)	1.67 (0.43; 2.92)	0.07 (-0.10; 0.24)	0.13 (-0.37; 0.64)	Year	0.04 (-0.36; 0.44)	-0.67 (-1.83; 0.46)	-0.93 (-2.59; 0.75)	1.91 (0.86; 2.95)	-0.09 (-0.34; 0.14)	0.41 (-0.09; 0.91)
Date[d]	-0.15 (-0.36; 0.06)	-0.32 (-0.85; 0.18)	-0.02 (-0.38; 0.42)	-0.61 (-1.27; 0.02)	-0.05 (-0.13; 0.03)	-0.20 (-0.45; 0.05)	Date[d]	-0.15 (-0.35; 0.07)	-0.31 (-0.81; 0.19)	-0.01 (-0.40; 0.41)	-0.61 (-1.16; -0.07)	-0.05 (-0.12; 0.03)	-0.15 (-0.39; 0.09)
Cumulative rainfall * Sex	-0.04 (-0.31; 0.23)	0.04 (-0.76; 0.84)	-0.15 (-0.58; 0.26)	-0.18 (-1.13; 0.74)	0.08 (-0.02; 0.18)	0.00 (-0.32; 0.34)	Mean cumulative rainfall * Sex	-0.06 (-0.27; 0.15)	-0.11 (-0.81; 0.59)	-0.20 (-0.63; 0.16)	-0.63 (-1.22; -0.06)	-0.07 (-0.16; -0.03)	-0.26 (-0.51; 0.01)
Cumulative rainfall * Year	0.16 (-0.22; 0.54)	0.95 (-0.29; 2.17)	-0.68 (-1.84; 0.48)	-0.26 (-1.88; 1.36)	0.25 (0.03; 0.48)	-0.37 (-0.97; 0.24)	Mean cumulative rainfall * Year	0.08 (-0.32; 0.49)	-0.09 (-1.23; 1.02)	0.21 (-1.52; 1.93	1.04 (-0.15; 2.24)	-0.12 (-0.38; 0.14)	0.29 (-0.23; 0.82)
Random factors σ^2 (95% CrI)													
Band number	0.29 (0.24; 0.37)	1.39 (0.98; 1.94)	-	-	0.01 (0.01; 0.02)	0.35 (0.25; 0.48)	Band number	0.19 (0.14; 0.24)	1.79 (1.28; 2.46)	-	5.47 (4.46; 6.79)	-	0.43 (0.31; 0.57)
Nest ID	-	0.25 (0.15; 0.38)	0.84 (0.59; 1.16)	0.22 (0.13; 0.34)	0.02 (0.01; 0.03)	0.18 (0.12; 0.27)	Nest ID	0.11 (0.07; 0.16)	0.03 (0.02; 0.05)	0.88 (0.64; 1.19)	0.60 (0.38; 0.89)	0.03 (0.02; 0.04)	0.16 (0.10; 0.23)
Residual variance	0.09 (0.07; 0.12)	1.75 (1.29; 2.33)	0.71 (0.52; 0.98)	4.97 (3.72; 6.69)	0.04 (0.03; 0.05)	0.23 (0.18; 0.32)	Residual variance	0.11 (0.08; 0.15)	1.87 (1.40; 2.47)	0.69 (0.51; 0.93)	0.00 (0.00; 0.00)	0.05 (0.04; 0.07)	0.16 (0.12; 0.21)

9.2. Supplementary information chapter 2: figure

Figure 1. Pearson correlation coefficients and p-values indicated within brackets for all physiological traits measured during the breeding seasons of 2015 and 2016. For all traits, the pair-wise correlations were computed using the residuals of each physiological variable and current ambient temperature. Color indicates strength of correlations, with blue indicating positive and red indicating negative relationships. Asterisks indicate p-values smaller than 0.05. Oxidative status parameters: OXY, non-enzymatic antioxidants in plasma; GPX, enzymatic antioxidant in red blood cells; ROMs, reactive oxygen metabolites in plasma.



9.3. Supplementary information chapter 3: tables

Table 1. Results from linear mixed-effects models estimating fixed and random effects explaining variation in egg and yolk mass. Egg position was fitted as a covariate, and random slopes were fitted for female identity with respect to egg position. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets. All explanatory variables were mean centered; hence the intercept refers to the average value of covariates. Fixed factors with a statistically meaningful effect (i.e., if zero is not included within the 95% CrI) is presented in bold. Estimates and CrI of '0.00' represent an effect smaller than 0.01.

	Egg mass	Yolk mass
Fixed factors β (95% CrI)		
Intercept	1.60 (1.53; 1.68)	0.29 (0.27; 0.30)
Egg position[a]	**0.04 (0.01; 0.06)**	0.01 (-0.00; 0.01)
Random factors σ^2 (95% CrI)		
Among-female variance		
V elevation[b]	0.02 (0.01; 0.02)	0.00 (0.00; 0.00)
V slopes[c]	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)
Cor elevation-slopes[d]	-0.31 (-0.59; 0.003)	-0.32 (-0.67; 0.12)
Residual variance	0.00 (0.00; 0.01)	0.00 (0.00; 0.00)

[a] Egg number corrected by total clutch size.

[b] Total amount of variation in reaction norm elevation among-females.

[c] Total amount of variation in reaction norm slopes among-females.

[d] Elevation-slope correlation.

Table 2. Mean concentrations of steroid hormones and antioxidants, and mean proportions of groups of fatty acids in great tit egg yolks from 11 clutches. The total number of eggs analyzed per component is indicated within brackets.

Mean ± SE (*n*)	
Steroid hormone concentrations (pg/mg)	
Androstenedione	21.86 ± 0.73 (93)
5α-dihydrotestosterone	12.82 ± 0.45 (93)
Testosterone	22.21 ± 0.96 (93)
Corticosterone	0.17 ± 0.01 (93)
Antioxidant concentrations (standard micromolar)[a]	
Vitamin E	1.34 ± 0.09 (92)
Lutein	133.32 ± 11.52 (92)
Zeaxanthin	6.40 ± 0.61 (90)
Ratio Lutein/Zeaxanthin	23.65 ± 0.86 (90)
Carotenoids	139.48 ± 12.13 (90)
% of total fatty acid content[b]	
SFA	27.88 ± 0.25 (93)
MUFA	46.10 ± 0.14 (93)
ω-3 PUFA	3.81 ± 0.22 (93)
ω-6 PUFA	22.21 ± 0.38 (93)
Ratio ω-6/ ω-3 PUFA	7.11 ± 0.28 (93)

[a] Vitamin E, α-tocopherol; Carotenoids, sum of lutein and zeaxanthin.

[b] SFA, saturated fatty acids; MUFA monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 3. Overall relative abundance (% of total fatty acid content) and classification of fatty acids in great tit egg yolks.

Fatty acid[a]	**C:Dn-x[b]**	**Fatty acid group[c]**	**Mean % ± SE (*n* = 93)**
Myristic acid	14:0	SFA	0.44 ± 0.03
Pentadecanoic acid	15:0	SFA	0.06 ± 0.002
Palmitic acid	16:0	SFA	19.11 ± 0.28
Margaric acid	17:0	SFA	0.47 ± 0.01
Stearic acid	18:0	SFA	7.80 ± 0.08
Myristoleic acid	14:1	MUFA	0.02 ± 0.004
Hexadecenoic acid	16:1n-9	MUFA	1.34 ± 0.03
Palmitoleic acid	16:1n-7	MUFA	1.09 ± 0.06
Oleic acid	18:1n-9	MUFA	40.92 ± 0.15
cis-Vaccenic acid	18:1n-7	MUFA	1.27 ± 0.03
Eicosenoic acid	20:1n-9	MUFA	1.44 ± 0.05
α-Linolenic acid	18:3n-3	ω-3 PUFA	2.22 ± 0.19
Eicosapentaenoic acid	20:5n-3	ω-3 PUFA	0.26 ± 0.01
Docosapentaenoic acid	22:5n-3	ω-3 PUFA	1.07 ± 0.04
Docosahexaenoic acid	22:6n-3	ω-3 PUFA	0.26 ± 0.01
Linoleic acid	18:2n-6	ω-6 PUFA	19.66 ± 0.34
Eicosadienoic acid	20:2n-6	ω-6 PUFA	0.28 ± 0.01
dihomo-ϒ-Linolenic acid	20:3n-6	ω-6 PUFA	0.25 ± 0.005
Arachidonic acid	20:4n-6	ω-6 PUFA	1.86 ± 0.04
Docosapentaenoic acid	22:5n-6	ω-6 PUFA	0.16 ± 0.01

[a] Trivial names of fatty acids are given if commonly used.

[b] C:Dn-x, number of carbon atoms:number of double bonds and position.

[c] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 4. Results from linear mixed-effects models estimating fixed and random effects explaining variation in yolk components and the variation among females. Egg position, mean ambient temperature and female body condition were fitted as covariates, and random slopes were fitted for female identity with respect to egg position. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI). All explanatory variables were mean centered; hence the intercept refers to the average value of covariates. Fixed factors with a statistically meaningful effect (i.e., if zero is not included within the 95% CrI) are given in bold font. Estimates and CrI of '0.00' represent an effect smaller than 0.01.

[a] A4, androstenedione; DHT, 5α-dihydrotestosterone; Testo, testosterone; Cort, corticosterone.

Concentrations of A4, DHT and Testo were square root transformed.

[b] Vit E, vitamin E (α-tocopherol); Lut, lutein; Zea, zeaxanthin; Carot, sum of lutein and zeaxanthin.

Concentrations of Vit E and Carot were square root transformed; Zea was $\log_{10}$ transformed.

[c] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

All fatty acid proportions were logit transformed; the total ω-6/total ω-3 PUFA ratios were $\log_{10}$ transformed.

[d] Egg number corrected by the clutch size.

[e] Mean ambient temperature for the 3 days prior to the lay date of each egg.

[f] Scaled body mass index.

[g] Total amount of variation in reaction norm elevation among-females.

[h] Total amount of variation in reaction norm slopes among-females.

[i] Elevation-slope correlation.

	Steroid hormones[a]				**Antioxidants[b]**				**Fatty acids[c]**				
	A4	DHT	Testo	Cort	Vit E	Lut	Zea	Carot	SFA	MUFA	ω-3 PUFA	ω-6 PUFA	ω-6/ω-3 PUFA
Fixed factors β (95% CrI)													
Intercept	4.58 (4.28; 4.89)	3.55 (3.32; 3.78)	4.73 (4.38; 5.06)	0.18 (0.15; 0.21)	1.01 (0.89; 1.14)	118.94 (88.16; 149.36)	1.21 (0.84; 1.57)	10.22 (8.73; 11.72)	-0.97 (-1.03; -0.92)	-0.15 (-0.18; -0.13)	-3.42 (-3.56; -3.29)	-1.21 (-1.28; -1.14)	1.99 (1.81; 2.16)
Egg position[d]	-0.02 (-0.16; 0.12)	**-0.17 (-0.29; -0.04)**	0.09 (-0.10; 0.26)	-0.00 (-0.03; 0.02)	**-0.16 (-0.24; -0.08)**	**-32.21 (-58.31; -6.53)**	**-0.29 (-0.55; -0.04)**	**-1.55 (-2.59; -0.52)**	**0.03 (0.00; 0.05)**	0.01 (-0.00; 0.02)	0.04 (-0.02; 0.09)	**-0.05 (-0.08; -0.01)**	-0.07 (-0.15; 0.00)
Mean ambient temperature[e]	0.03 (-0.13; 0.19)	0.09 (-0.00; 0.17)	0.14 (-0.07; 0.34)	**-0.03 (-0.05; -0.01)**	0.01 (-0.08; 0.09)	0.75 (-21.42; 23.38)	0.02 (-0.26; 0.30)	0.06 (-1.04; 1.15)	-0.00 (-0.01; 0.01)	**-0.01 (-0.02; -0.00)**	-0.02 (-0.05; 0.01)	**0.02 (0.00; 0.03)**	0.03 (-0.01; 0.07)
Female body condition[f]	-0.18 (-0.48; 0.14)	**-0.30 (-0.53; -0.05)**	-0.24 (-0.58; 0.09)	0.02 (-0.01; 0.05)	**0.15 (0.03; 0.26)**	17.29 (-8.38; 43.30)	0.22 (-0.16; 0.59)	0.78 (-0.64; 2.19)	-0.00 (-0.05; 0.05)	0.02 (-0.00; 0.04)	-0.03 (-0.17; 0.11)	-0.02 (-0.08; 0.04)	0.04 (-0.14; 0.21)
Random factors σ^2 (95% CrI)													
Among-female variance													
V elevation[g]	0.16 (0.08; 0.31)	0.11 (0.07; 0.18)	0.18 (0.07; 0.36)	0.00 (0.00; 0.00)	0.02 (0.01; 0.04)	1714.79 (603.69; 3655.31)	0.17 (0.06; 0.36)	3.27 (1.15; 6.98)	0.01 (0.00; 0.01)	0.00 (0.00; 0.00)	0.04 (0.03; 0.07)	0.01 (0.01; 0.02)	0.06 (0.05; 0.11)
V slopes[h]	0.06 (0.05; 0.13)	0.02 (0.02; 0.07)	0.08 (0.07; 0.14)	0.00 (0.00; 0.00)	0.01 (0.01; 0.02)	997.40 (942.53; 1179.50)	0.09 (0.09; 0.15)	1.80 (1.73; 2.59)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.01 (0.01; 0.03)	0.00 (0.00; 0.00)	0.01 (0.01; 0.05)
Cor elevation-slopes[i]	**-0.84 (-0.96; -0.60)**	0.44 (-0.02; 0.77)	**-0.79 (-0.95; -0.51)**	-0.41 (-0.81; 0.12)	**-0.70 (-0.93; -0.29)**	**-0.84 (-0.97; -0.59)**	**-0.69 (-0.93; -0.26)**	**-0.71 (-0.93; -0.31)**	0.35 (-0.21; 0.75)	**-0.71 (-0.93; -0.27)**	-0.07 (-0.44; 0.33)	**0.52 (0.05; 0.79)**	0.01 (-0.41; 0.42)
Residual variance	0.38 (0.27; 0.53)	0.09 (0.07; 0.13)	0.46 (0.65; 0.90)	0.00 (0.00; 0.01)	0.12 (0.09; 0.17)	6324.37 (4540.44; 8786.69)	1.19 (0.85; 1.66)	17.33 (12.47; 23.94)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.01 (0.01; 0.02)	0.00 (0.00; 0.00)	0.02 (0.01; 0.02)

9.4. Supplementary information chapter 4: materials and methods

Correlations between yolk components

The correlation between yolk components was determined with pairwise Pearson's analysis (Supplementary information 9.6, Figure 1). For this, all variables were mean-centered. We corrected for multiple tests via false discovery rate (i.e., 'fdr'). Statistical support was obtained from p-values.

Relationships between nestling tarsus and survival

To understand which nestling phenotypic traits were associated with nestling survival, we ran linear mixed effect models with a Poisson distribution. Although we studied the relationship of each phenotypic trait (i.e., morphological and physiological traits) with survival, since this was a complementary analysis to better understand our results, here we only present the models for the relationship between survival and tarsus length. We first fitted survival from day 6 to day 12 (i.e., from early development to the period when chicks show exponential growth) as the response variable. Then, we fitted survival from day 12 to day 15 (i.e., until the time when nestlings were about to fledge). Tarsus length (either on day 6 or 12), clutch size and date were included as covariates, and nest ID was included as a random factor.

Relationships between nestling body condition and oxidative status

To better interpret the physiological condition of the chicks on day 12, we analyzed the relationships between each morphological and physiological parameter measured. For this, we ran linear mixed effect models fitting OXY, GXP and ROMs as response variables. In separate models, mass, tarsus length (results not shown) and growth rate were added as covariates together with date of capture and clutch size. All covariates were mean-centered. Nest ID was included as a random factor.

Relationship between yolk androstenedione and fledgling success

We ran linear mixed models with a Poisson distribution to study the relationship between androstenedione and fledging success. Fledging success was the response variable, and androstenedione, clutch size and date of capture were fitted as covariates. All covariates were mean centered.

Results from linear mixed effect models were considered to be ‘statistically meaningful’ when the posterior probability of the mean difference between compared estimates was higher than 0.95. Further information on the statistical approach used can be found in the main text (in Materials and Methods section). Results for each of the models are presented in Supplementary Tables 6, 7, 8 & 9.

9.5. Supplementary information chapter 4: tables

Table 1. Principal components analysis (PCA) of 31 yolk components measured in the fourth egg of 69 wild great tit clutches. The yolk components with highest loadings in a given PC are presented in bold.

Yolk components	Yolk groups	PC1	PC2	PC3
Eigenvectors				
Androstenedione	Steroid hormones	-0.13	0.13	**0.28**
5α-dihydrotestosterone		0.02	0.07	**0.43**
Testosterone		-0.00	0.07	**0.30**
Corticosterone		0.11	0.03	0.15
Vitamin E	Antioxidants	**-0.21**	0.02	0.05
Lutein		0.14	-0.14	**0.32**
Zeaxanthin		0.15	-0.03	**0.31**
Lauric acid (12:0)	Saturated fatty acids (SFA)	0.04	0.14	0.16
Myristic acid (14:0)		0.10	0.18	0.10
Pentadecanoic acid (15:0)		-0.02	**0.32**	0.14
Palmitic acid (16:0)		0.09	**0.40**	0.04
Margaric acid (17:0)		-0.13	**0.20**	0.13
Stearic acid (18:0)		-0.18	**0.28**	-0.19
Oleic acid (18:1n-9)	Monounsaturated fatty acids (MUFA)	0.14	**0.28**	**-0.28**
Hexadecenoic acid (16:1n-9)		0.05	**0.24**	**-0.31**
Palmitoleic acid (16:1n-7)		0.11	**0.20**	-0.03
cis-Vaccenic acid (18:1n-7)		-0.18	**0.24**	-0.03
Eicosenoic acid (20:1n-9)		**-0.22**	0.05	-0.11
α-Linolenic acid (18:3n-3)	ω-3 Polyunstaturated fatty acid (ω-3 PUFA)	0.18	**0.28**	0.13
Eicosapentaenoic acid (20:5n-3)		0.01	**0.28**	0.13
Docosapentaenoic acid (22:5n-3)		0.09	**0.25**	-0.08
Docosahexaenoic acid (22:6n-3)		-0.02	**0.25**	0.03
γ-linolenic acid (18:3n-6)	ω-6 Polyunstaturated fatty acid (ω-6 PUFA)	**-0.27**	0.08	0.03
Hexadecadienoic acid (16:2n-6)		**-0.29**	0.03	0.08
Linoleic acid (18:2n-6)		**-0.29**	0.08	0.01
Pinolenic acid (18:3n-6)		**-0.29**	-0.01	0.13
Eicosadienoic acid (20:2n-6)		**-0.28**	-0.08	0.01
dihomo- γ -Linolenic acid (20:3n-6)		**-0.28**	-0.02	0.08
Arachidonic acid (20:4n-6)		**-0.22**	0.07	-0.17
Adrenic acid (22:4n-6)		**-0.23**	0.02	-0.07
Eigenvalue				
Standard deviation		3.28	2.09	1.69
% Total variance		34.69	14.08	9.31
% Cumulative variance		34.69	48.77	58.08

Table 2. Linear models testing for differences in yolk composition (as represented by PC1, PC2 and P3) between years. Year (2015 vs 2016) was fitted as a fixed factor. We present fixed (β) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates is higher than 0.95) are presented in bold.

	PC1 [a]	PC2 [b]	PC3 [c]
Fixed factors β (95% CrI)			
Intercept	3.41 (2.07; 4.78)	-0.02 (-1.08; 1.02)	-1.07 (-1.88; -0.25)
Year	**-4.46** **(-6.01; -2.93)**	0.03 (-1.19; 1.22)	**1.38** **(0.47; 2.29)**

[a] PC1 was mainly represented by low concentrations of vitamin E (α - tocopherol) and ω-6 polyunsaturated fatty acids (PUFAs).

[b] PC2 was mainly represented by high concentrations of saturated (SFA), monounsaturated (MUFA) and all ω-3 PUFAs.

[c] PC3 was mainly represented by high concentrations of androgens (androstenedione, 5α-dihydrotestosterone and testosterone) and carotenoids (lutein and zeaxanthin).

Table 3. Results of linear models and linear-mixed effects models to test for relationships between yolk composition and fitness traits. PC1, PC2, PC3, date and clutch size were included as covariates. All covariates were mean-centered. Nest ID was included as a random factor in the linear mixed effect models. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates was higher than 0.95) are presented in bold.

[a] PC1 was mainly represented by low concentrations of vitamin E (α - tocopherol) and ω-6 polyunsaturated fatty acids (PUFAs).

[b] PC2 was mainly represented by high concentrations of saturated (SFA), monounsaturated (MUFA) and all ω-3 PUFAs.

[c] PC3 was mainly represented by high concentrations of androgens (androstenedione, 5α-dihydrotestosterone and testosterone) and carotenoids (lutein and zeaxanthin).

[d] Date when the fourth egg was collected.

	Clutch size	Hatchling number	Fledgling number	Fledgling mass corrected for clutch size	Fledgling tarsus corrected for clutch size
Fixed factors β (95% CrI)					
Intercept	2.02 (1.93; 2.10)	1.46 (1.35; 1.57)	0.73 (0.55; 0.91)	0.03 (-0.51; 0.56)	0.01 (-0.19; 0.22)
PC1[a]	-0.03 (-0.11; 0.07)	-0.03 (-0.15; 0.08)	**0.19 (0.03; 0.34)**	-0.19 (-0.78; 0.39)	-0.09 (-0.31; 0.14)
PC2[b]	0.03 (-0.06; 0.11)	**0.10 (-0.01; 0.21)**	**0.29 (0.17; 0.43)**	0.13 (-0.47; 0.73)	-0.18 (-0.05; 0.42)
PC3[c]	0.02 (-0.08; 0.11)	-0.08 (-0.20; 0.06)	0.02 (-0.16; 0.19)	0.10 (-0.49; 0.69)	0.05 (-0.18; 0.28)
Date[d]	-0.05 (-0.15; 0.06)	-0.01 (-0.15; 0.13)	**-0.46 (-0.75; -0.15)**	-0.08 (-0.55; 0.38)	0.02 (-0.17; 0.21)
Clutch size	-	0.07 (-0.06; 0.19)	**-0.21 (-0.40; -0.02)**	-	-
Random factors σ^2 (95% CrI)					
Nest ID	-	-	-	2.25 (1.69; 2.94)	0.27 (0.18; 0.38)
Residual variance	-	-	-	1.57 (1.26; 1.97)	0.52 (0.41; 0.65)

Table 4. Results of linear-mixed effects models to test the relationship between yolk composition and phenotypic traits on nestling day 6. PC1, PC2, PC3, date and clutch size were included as covariates. All covariates were mean-centered. Nest ID was included as a random factor in the linear mixed effect models. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates was higher than 0.95) are presented in bold.

[a] PC1 was mainly represented by low concentrations of vitamin E (α - tocopherol) and ω-6 polyunsaturated fatty acids (PUFAs).

[b] PC2 was mainly represented by high concentrations of saturated (SFA), monounsaturated (MUFA) and all ω-3 PUFAs.

[c] PC3 was mainly represented by high concentrations of androgens (androstenedione, 5α-dihydrotestosterone and testosterone) and carotenoids (lutein and zeaxanthin).

[d] Date when the fourth egg was collected.

[e] Non-enzymatic antioxidant measured in plasma.

[f] Oxidative damage compounds measured in plasma.

[g] Calculated as [mass on day 6 – mass on day 1]/[day 6 – day 1].

	OXY [e]	ROMs [f]	Nestling mass corrected for clutch size	Nestling tarsus corrected for clutch size	Growth rate[g]
Fixed factors β (95% CrI)					
Intercept	223.17 (204.08; 241.92)	1.12 (1.00; 1.25)	-0.02 (-0.39; 0.35)	-0.03 (-0.31; 0.26)	1.26 (1.19; 1.33)
PC1 [a]	-11.40 (-30.96; 8.27)	-0.02 (-0.15; 0.11)	-0.11 (-0.48; 0.26)	-0.11 (-0.38; 0.17)	-0.03 (-0.10; 0.04)
PC2 [b]	-4.32 (-25.94; 17.56)	0.05 (-0.08; 0.19)	0.20 (-0.15; 0.56)	**0.24 (-0.01; 0.50)**	0.04 (-0.03; 0.11)
PC3 [c]	**-17.77 (-37.40; 1.93)**	0.05 (-0.08; 0.18)	0.07 (-0.35; 0.47)	0.07 (-0.22; -.37)	0.01 (-0.06; 0.08)
Date [d]	-7.23 (-27.36; 13.12)	-0.07 (-0.21; 0.05)	-0.12 (-0.49; 0.25)	-0.15 (-0.43; 0.13)	0.00 (-0.06; 0.06)
Clutch size	**-19.59 (-39.91; -0.01)**	-	-	-	**-0.07 (-0.14; -0.01)**
Random factors σ^2 (95% CrI)					
Nest ID	3311.55 (2400.79; 4389.16)	0.12 (0.08; 0.16)	1.57 (1.19; 2.02)	0.77 (0.56; 1.02)	0.04 (0.03; 0.06)
Residual variance	3749.83 (3051.48; 4623.26)	0.26 (0.21; 0.32)	1.65 (1.38; 1.97)	1.34 (1.18; 1.59)	0.04 (0.03; 0.05)

Table 5. Results of linear-mixed effects models to test the relationship between yolk composition and phenotypic traits on day 12. PC1, PC2, PC3, date and clutch size were included as covariates. All covariates were mean-centered. Nest ID was included as a random factor in the linear mixed effect models. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates is higher than 0.95) are presented in bold.

[a] PC1 was mainly represented by low concentrations of vitamin E (α - tocopherol) and ω-6 polyunsaturated fatty acids (PUFAs).

[b] PC2 was mainly represented by high concentrations of saturated (SFA), monounsaturated (MUFA) and all ω-3 PUFAs.

[c] PC3 was mainly represented by high concentrations of androgens (androstenedione, 5α-dihydrotestosterone and testosterone) and carotenoids (lutein and zeaxanthin).

[d] Date when the fourth egg was collected.

[e] Time since we arrived at the nest until we finished to take blood to each individual

[f] Non-enzymatic antioxidant measured in plasma.

[g] Enzymatic antioxidant measured in red blood cells.

GPX was squared root transformed.

[h] Oxidative damage compounds measured in plasma.

ROMs was log10 transformed.

[i] Calculated as [mass on day 12 – mass on day 1]/[day 12 – day 1].

	OXY [f]	GPX [g]	ROMs [h]	Nestling mass corrected for clutch size	Nestling tarsus corrected for clutch size	Growth rate [i]
Fixed factors β (95% CrI)						
Intercept	221.58 (208.60; 234.86)	1.54 (1.23; 1.84)	0.27 (0.15; 0.38)	0.08 (-0.58; 0.75)	0.03 (-0.30; 0.36)	1.07 (1.01; 1.13)
PC1 [a]	1.30 (-12.47; 15.36)	**0.37 (0.06; 0.68)**	**-0.12 (-0.24; 0.00)**	-0.30 (-1.01; 0.41)	-0.19 (-0.54; 0.16)	-0.02 (-0.08; 0.04)
PC2 [b]	**-11.81 (-26.54; 2.85)**	-0.09 (-0.40; -0.21)	0.07 (-0.04; 0.19)	0.04 (-0.53; 0.61)	0.12 (-0.17; 0.39)	0.00 (-0.06; 0.07)
PC3 [c]	**-11.43 (-25.02; 1.87)**	-0.04 (-0.35; 0.26)	-0.01 (-0.13; 0.11)	-0.34 (-1.07; 0.40)	-0.05 (-0.43; 0.33)	-0.02 (-0.08; 0.04)
Date [d]	1.56 (-12.67; 15.55)	0.21 (-0.10; 0.51)	-0.01 (-0.13; 0.11)	-0.36 (-0.92; 0.21)	-0.25 (-0.54; 0.05)	-0.01 (-0.06; 0.03)
Clutch size	-9.25 (-24.53; 5.81)	-	-	-	-	**-0.06 (-0.12; -0.00)**
Total sampling time [e]	-	-	**-0.08 (-0.16; 0.00)**	-	-	-
Random factors σ^2 (95% CrI)						
Nest ID	519.85 (295.15; 820.63)	0.39 (0.24; 0.59)	0.07 (0.05; 0.11)	3.22 (2.44; 4.27)	0.76 (0.56; 1.03)	0.21 (0.02; 0.03)
Residual variance	2659.98 (1956.52; 3611.16)	1.17 (0.87; 1.57)	0.12 (0.09; 0.17)	1.87 (1.49; 2.33)	0.71 (0.57; 0.89)	0.01 (0.01; 0.02)

Table 6. Results of linear-mixed effects models used to test the relationship between nestling tarsus length on day 6 and 12 and survival in the nest. Tarsus length, clutch size and date were included as covariates. All covariates were mean-centered. Nest ID was included as a random factor in the linear mixed effect models. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates is higher than 0.95) are presented in bold.

	Survived (day 6 to day 12) [c]	Survived (day 12 to day 15) [d]
Fixed factors β (95% CrI)		
Intercept	7.82 (2.85; 12.79)	4.87 (2.83; 6.89)
Tarsus [a]	**1.86** **(0.81; 2.94)**	**1.35** **(0.34; 2.35)**
Clutch size	0.32 (-4.09; 4.69)	-0.68 (-2.15; 0.78)
Date [b]	-0.74 (-4.14; 2.64)	**-0.75** **(1.58; 0.06)**
Random factors σ^2 (95% CrI)		
Nest ID	116.66 (70.74; 175;13)	-
Residual variance	0.99 (0.99; 0.99)	-

[a] Nestling tarsus length measured on day 6 and day 12 (first and second models, respectively).

[b] Date when the fourth egg was collected.

[c] Nestlings measured on day 6 (i.e., from early development) that survived to day 12 (i.e., period when chicks have an exponential grow).

[d] Nestlings measured on day 12 that survived to day 15 (i.e., when nestlings were about to fledge).

Table 7. Results of the linear model used to test the relationship between the concentration of yolk androstenedione and fledging success. Androstenedione, clutch size and date were included as covariates. All covariates were mean centered. We present fixed (β) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates is higher than 0.95) are presented in bold.

	Fledging success
Fixed factors β (95% CrI)	
Intercept	0.75 (0.57; 0.93)
Androstenedione	**0.22** **(0.08; 0.36)**
Clutch size	-0.06 (-0.25; 0.12)
Date [a]	**-0.51** **(-0.82; -0.21)**

[a] Date when the fourth egg was collected.

Table 8. Results of linear-mixed effects models used to test the relationship between nestling mass and oxidative stress markers on day 12. Mass, clutch size and date were included as covariates. All covariates were mean centered. Nest ID was included as a random factor in the linear mixed effect models. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates is higher than 0.95) are presented in bold.

	OXY [b]	GPX [c]	ROMs [d]
Fixed factors β (95% CrI)			
Intercept	221.85 (207.95; 235.78)	3.98 (2.74; 5.24)	1.43 (1.23; 1.63)
Mass	1.43 (-12.05; 14.91)	0.25 (-0.91; 1.47)	**0.23 (0.07; 0.39)**
Clutch size	-11.32 (-26.13; 3.20)	-0.65 (-1.97; 0.67)	0.06 (-0.15; 0.26)
Date [a]	-11.32 (-13.70; 14.61)	0.23 (-1.03; 1.49)	-0.00 (-0.19; 0.19)
Random factors σ^2 (95% CrI)			
Nest ID	674.80 (397.30; 1056.19)	5.38 (3.19; 8.27)	0.25 (0.17; 0.35)
Residual variance	2705.74 (1996.74; 3654.70)	22.80 (17.09; 30.50)	0.24 (0.17; 0.32)

[a] Date when the fourth egg was collected.

[b] Non-enzymatic antioxidant measured in plasma.

[c] Enzymatic antioxidant measured in red blood cells.

[d] Oxidative damage compounds measured in plasma.

Table 9. Results of linear-mixed effects models used to test the relationship between nestling growth rate and oxidative stress markers on day 12. Growth rate, clutch size and date were included as covariates. All covariates were mean-centered. Nest ID was included as a random factor in the linear mixed effect models. We present fixed (β) and random (σ^2) parameters with their 95% credible intervals (CrI) in brackets. Fixed factors with a statistically meaningful effect (i.e., if the mean difference between compared estimates was higher than 0.95) are presented in bold.

	OXY [c]	GPX [d]	ROMs [e]
Fixed factors β (95% CrI)			
Intercept	219.09 (205.55; 232.82)	4.09 (2.90; 5.29)	1.43 (1.24; 1.63)
Growth rate [a]	2.23 (-11.53; 15.87)	-0.59 (-1.8-; 0.59)	**0.25 (0.08; 0.42)**
Clutch size	-10.22 (-24.71; 3.92)	-0.91 (-2.15; 0.33)	0.06 (-0.14; 0.26)
Date [b]	1.16 (-12.67; 15.09)	0.24 (-0.97; 1.46)	-0.02 (-0.21; 0.17)
Random factors σ^2 (95% CrI)			
Nest	486.54 (274.32; 774.89)	3.57 (2.02; 5.72)	0.22 (0.15; 0.32)
Residual variance	2776.51 (2042.67; 3738.65)	24.21 (17.97; 32.78)	0.24 (0.18; 0.32)

[a] Calculated as [mass on day 12 – mass on day 1]/[day 12 – day 1].

[b] Date when the fourth egg was collected.

[c] Non-enzymatic antioxidant measured in plasma.

[d] Enzymatic antioxidant measured in red blood cells.

[e] Oxidative damage compounds measured in plasma.

9.6. Supplementary information chapter 4: figure

Figure 1. Correlation matrix heat map for yolk components measured in the fourth egg of wild great tits. A4, androstenedione; DHT, 5α-dihydrotestosterone; Testo, testosterone; Cort, corticosterone; Antioxidants: Carot, sum of lutein and zeaxanthin, Vit E, vitamin E (α-tocopherol); Fatty acids: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. All concentrations were mean-centered. Colors indicate strength of correlations, blue indicating positive and red indicating negative relationships. Correlations with p-values smaller than 0.001, 0.01, 0.05 are indicated with "***', "**', "*", respectively. The absence of stars indicates p-values bigger than 0.05.