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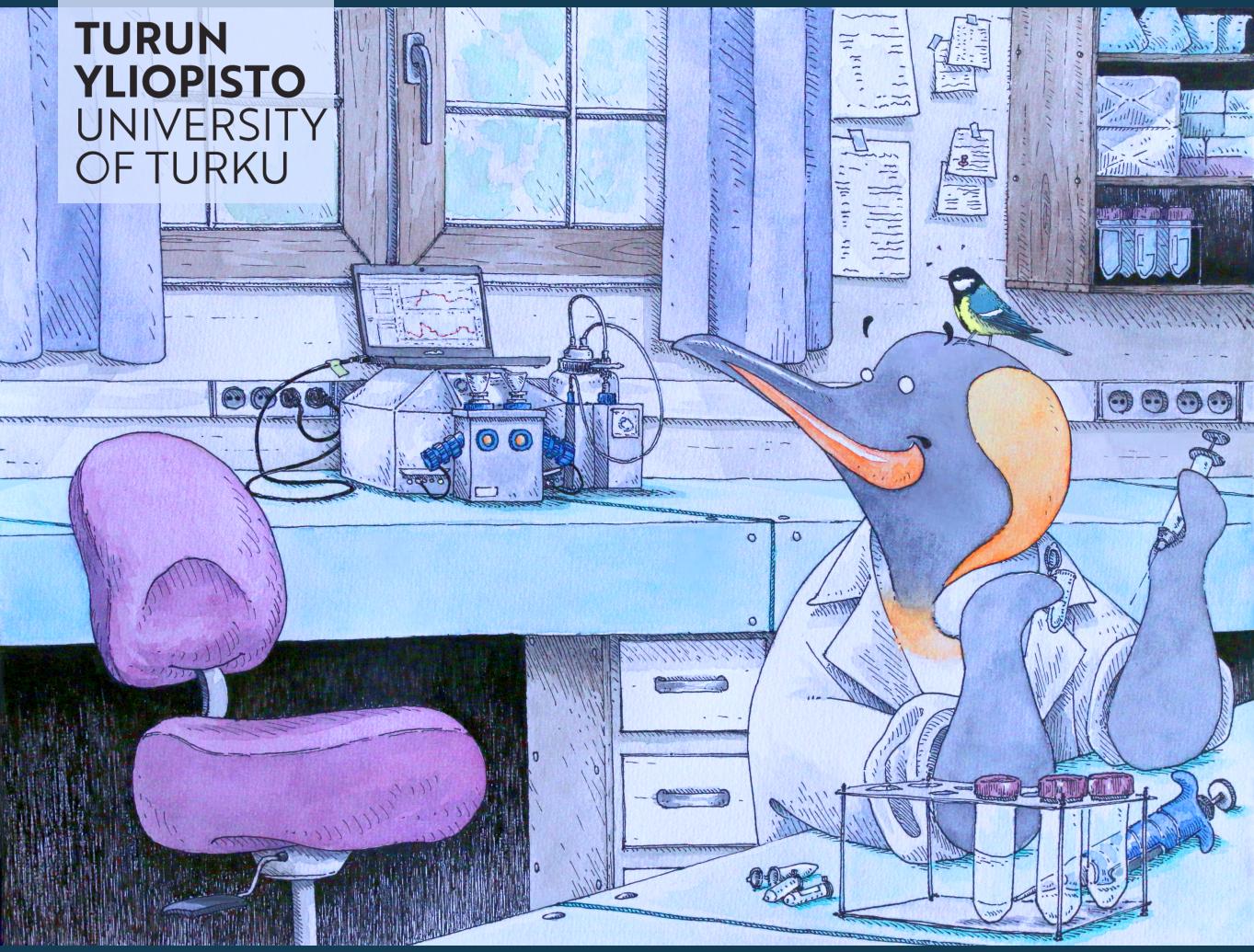

HOW CAN CELLULAR-LEVEL ENERGY PRODUCTION EXPLAIN HOW WILD BIRDS COPE WITH ENVIRONMENTAL STRESS?

Nina Cossin-Sevrin

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
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ABSTRACT:

All animals rely on energy in order to grow, reproduce and thrive. The conversion of nutrients into energy happens in mitochondria, which are crucial components of the cells. The efficiency with which mitochondria produce energy varies within species and is increasingly thought to play a fundamental role in explaining individual heterogeneity in growth, reproduction and survival. Indeed, mitochondrial metabolism and efficiency can vary through phenotypic plasticity in response to environmental factors such as temperature, food availability or any stressful stimulus. However, despite the importance of environmental determinants in metabolism, we still lack key information about wild species within their natural habitat. As a result, physiological capacities and potential responses to environmental changes are underappreciated, especially in birds. With this doctoral project, I explore how red blood cell mitochondrial metabolism and its efficiency vary in response to different environmental challenges in two distinct wild bird species (the Great tit and the King penguin) within their ecosystem and at various life stages (both during growth and adulthood). Here, I investigate the impact of early-life environmental adversity, nutritive stress and maternal effects on mitochondrial metabolism, and, in turn, its impact on individual growth and survival, in order to provide much-needed information for understanding how mechanistic constraints at a cellular level may shape the ecology and adaptability in two different avian species *in natura*.

KEYWORDS: Developmental plasticity, cellular energetics, mitochondrial respiration, maternal effects, early-life adversity, *Parus major*, *Aptenodytes patagonicus*

TURUN YLIOPISTO
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Biologian laitos
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TIIVISTELMÄ:

Kaikki eläimet tarvitsevat energiaa kasvaakseen, lisääntyäkseen ja menestyäkseen. Solutasolla ravinteiden muuntaminen energiaksi tapahtuu mitokondrioissa. Mitokondrioiden energiantuoton tehokkuus vaihtelee lajinsisäisesti ja sen on todettu selittävän yksilöidenvälisiä eroja kasvussa, lisääntymisessä ja selviytymisessä. Yksilöiden mitokondrioiden aineenvaihdunta ja tehokkuus voivat myös vaihdella fenotyyppisen plastisuuden kautta suhteessa ympäristötekijöihin, kuten lämpötilaan, ravinnonsaatavuuteen tai erilaisiin stressitekijöihin. Vaikka ympäristötekijöiden tiedetään vaikuttavan eläinten aineenvaihduntaan, tällä hetkellä on yhä puutteellisesti tietoa mitokondrioiden toiminnasta luonnonpopulaatioilla, ja erityisesti linnuilla siitä, miten solujen aineenvaihdunta reagoi ympäristönmuutoksiin. Tutkin väitöskirjassani, miten veren punasolujen mitokondriaalinen aineenvaihdunta ja sen tehokkuus vaihtelevat suhteessa erilaisiin ympäristöhaasteisiin kahdella luonnonvaraisella lintulajilla (talitiaisella ja kuningaspingviinillä) eri elinkierron vaiheissa (sekä kasvun että aikuisuuden aikana). Tutkin etenkin varhaiskehityksen ympäristön, erityisesti ravinnonpuutteen ja äitivaikutusten, merkitystä mitokondriaaliseen aineenvaihduntaan, ja sitä kautta miten ne ovat mahdollisesti yhteydessä yksilön kasvuun ja selviytymiseen. Väistökirjatutkimukseni antaa erityisesti tietoa siitä, miten luonnonvaraisilla linnuilla muutokset aineenvaihdunnassa solutasolla voivat muokata lajien ekologiaa ja sopeutumista.

ASIASANAT: Kehityksen plastisuus, solun energia-aineenvaihdunta, mitokondriaalinen metabolia, äitivaikutukset, varhaiskehityksen stressi, *Parus major*, *Aptenodytes patagonicus*

UNIVERSITÉ DE TURKU
Faculté des Sciences
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NINA COSSIN-SEVRIN: Comment le stress environmental module-t-il la respiration mitochondriale chez deux espèces d'oiseaux sauvages ?
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RÉSUMÉ *:*

C'est grâce à l'action des mitochondries que les cellules peuvent transformer les nutriments provenant de l'alimentation en énergie, un phénomène appelé la respiration mitochondriale. Cette énergie est essentielle pour permettre aux animaux de maintenir leurs fonctions vitales, pour grandir mais aussi se reproduire. Cependant, la capacité et l'efficacité de cette respiration mitochondriale varient entre individus, ce qui peut grandement impacter leur croissance mais aussi leur survie. Par exemple, certaines conditions environnementales telles que la température ambiante, ou la disponibilité alimentaire peuvent influencer la production d'énergie dans les cellules. Malgré son importance évidente pour tout organisme, l'influence de l'environnement sur la respiration mitochondriale reste peu étudiée sur les espèces sauvages dans leur habitat naturel. Le but de ma thèse est de mieux comprendre comment un environnement stressant peut influencer la respiration mitochondriale des oiseaux en milieu naturel. Pour cela, j'étudie deux espèces bien distinctes : la mésange charbonnière et le manchot royal. À travers ce projet, j'examine comment l'environnement en début de vie (effets maternels, stress nutritif) influence la respiration mitochondriale des globules rouges et étudie les potentielles conséquences sur la croissance et la survie des individus. Ce projet a pour but d'apporter plus de connaissances sur les possibles réponses physiologiques des oiseaux face à des environnements compromettants.

MOTS-CLEFS: Plasticité phénotypique, métabolisme cellulaire, respiration mitochondriale, effets maternels, *Parus major, Aptenodytes patagonicus*

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Abbreviations

ADP	Adenosine diphosphate
ANOVA	Analyses of Variance
ATP	Adenosine triphosphate
BMR	Basal metabolic rate
CI	Mitochondrial complex I: NADH dehydrogenase
CII	Mitochondrial complex II: Succinate dehydrogenase
CO	Control (group)
CORT	Corticosterone
ETS	Electron transport system
FCR	Flux Control Ratio
GCs	Glucocorticoids
GLM	Generalised linear model
GLMM	Generalised linear mixed-effects model
HSD	Tukey's Honest significance differences
HRR	High-resolution respirometry
ID	Identity
IPEV	Institut Paul Emile Victor (i.e. French Polar Institute)
LDA	Linear discriminant analyses
LEAK	Proton leak
LM	Linear mixed-effects model
mtDNAcn	Mitochondrial DNA copy number
OxCE	Oxidative phosphorylation coupling efficiency
OXPHOS	Oxidative phosphorylation

qPCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cell
RMR	Resting metabolic rate
ROS	Reactive oxygen species
ROUTINE	Endogenous mitochondrial respiration rate
SD	Standard deviation
SEM	Standard error of the mean
SUIT	Substrate-uncoupler-inhibitor titration (protocol)
TH	Thyroid hormones

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Cossin-Sevrin, N., Hsu, B. Y., Marciau, C., Viblanc, V. A., Ruuskanen, S., & Stier, A. Effect of prenatal glucocorticoids and thyroid hormones on developmental plasticity of mitochondrial aerobic metabolism, growth and survival: an experimental test in wild great tits. *Journal of Experimental Biology*, 225(9) (2022). https://doi.org/10.1242/jeb.243414.

II Cossin-Sevrin, N., Stier, A., Hukkanen, M., Zahn, S., Viblanc, V-A., Anttila, K., Ruuskanen, S. Early-life environmental effects on mitochondrial aerobic metabolism: a brood size manipulation in wild great tits. *Journal of Experimental Biology,* 226 (21) (2023). https://doi.org/10.1242/jeb.245932.

III Cossin-Sevrin, N., Bocquet, C., Lemonnier, C., Faulmann, T., Garcin, N., Lejeune, M., Bize, P., Robin, J-P., Anttila, K., Ruuskanen, S. & Viblanc, V-A. Sexual dimorphism in mitochondrial aerobic metabolism during breeding fasts in king penguins. (Under review in *Ecological and Evolutionary Physiology*)

IV Cossin-Sevrin, N., Anttila, K., Lejeune, M., Bocquet, C., Fusillier, M., Faulmann, T., Lemonnier, C., Garcin, N., Bize, P., Robin, J-P., Ruuskanen, S. & Viblanc, V-A. Early-life adversity modulates growth trajectories and mitochondrial aerobic metabolism in king penguin chicks. (Manuscript)

Author contributions to the original publications:

Original publications	Chapter I	Chapter II	Chapter III	Chapter IV
Conceptualization	B.Y.H., S.R., A.S.	N.C.S., S.R., A.S.	N.C.S., V-A.V.	J-P.R., V-A.V., P.B., N.C.S.
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Data curation	A.S., N.C.S.	N.C.S., S.Z.	N.C.S., C.B.	N.C.S., M.L., C.B.
Investigation	N.C.S., S.R., A.S.	N.C.S., S.R., K.A.	N.C.S., S.R., K.A.	N.C.S., S.R., K.A.
Statistical analysis	N.C.S.	N.C.S.	N.C.S.	N.C.S.
Writing the original draft of the manuscript	N.C.S., A.S.	N.C.S.	N.C.S.	N.C.S.
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1. Introduction

1.1 Phenotypic and developmental plasticity

1.1.1 Theory and concept in Evolution

The capacity of an animal species to reproduce and survive in a given environment is the result of the match between their phenotype and that environment. Phenotypic plasticity, described as the ability to express different phenotypes from one genotype in response to environmental conditions (Davis & Wund, 2016; Metcalfe, 2024; Piersma & Gils, 2011; West-Eberhard, 1989), is one mechanism allowing organisms to match their environment. In some cases, phenotypic plasticity occurs during ontogeny (i.e. developmental plasticity), and can (but not always) lead to an irreversible phenotype that is fixed according to early-life environmental conditions (Davis & Wund, 2016; Piersma & Gils, 2011; West-Eberhard, 2003, 2005).

However, the match between an organism's phenotype and its environment is not static: environments are changing (in a short- and long-term), and ever more rapidly under anthropogenic pressure, both at large scale (e.g. climate change) and local scale (e.g. introduced invasive species and habitat destruction). Recent research has shown that species may respond very differently to these changes (Howard et al., 2023). But even within a species, both phenotypes and responses to environmental change can vary considerably between individuals: part of this variation is genetic, but part

of it depends on non-genetic factors, and may, or may not, be inherited (Forsman, 2015; Laland et al., 2015; Müller, 2017; Pigliucci, 2007).

Phenotypic plasticity, including developmental plasticity, is increasingly seen as a central determinants of phenotypic variation, as is allows for rapid tracking of environmental conditions (Lind et al., 2020; Metcalfe, 2024; Uller, 2008). Interestingly, prior studies present evidence that individual plasticity can partially account for population responses to climate change (Bonamour et al., 2019; Charmantier et al., 2008; Donelson et al., 2023; Przybylo et al., 2000; Réale et al., 2003). Thus, exploring these sources of phenotypic variation and their consequences may ultimately help in understanding how species can cope with environmental challenges, and how this affects individuals fitness (Bonamour et al., 2019; Fox et al., 2019) and future population trajectories.

1.1.2 The determinants of developmental plasticity

Part of developmental plasticity is determined by genetic inheritance, including the ability of the genome to produce different phenotypes, but depends on complementary mechanisms as well (Jones & Robinson, 2018; Laland et al., 2015; Pigliucci, 2007; Pigliucci et al., 2006). Well-known examples have demonstrated how environmental conditions by themselves influence the offspring phenotype. For instance, in reptiles, the individual sex, morphology (e.g. body size and sexual maturity) and phenology (e.g. hatching date) are determined by the incubation temperature, which has been shown to affect the overall individual fitness (Kar et al., 2024; Noble et al., 2018; Warner & Shine, 2008). Another example is the effect of salinity levels on mosquito larval performance and abundance (abundance decreased with salinity) (Bengoa et al., 2022; Cordeschi et al., 2024; Rosenfeld et al., 2019). Another famous example is the caste determination (phenotype) by nutritional resources in honey bees (Haydak, 1970; Maleszka, 2018; Weiver, 1966). However, developmental plasticity is not always only influenced by the early-life “external” environmental conditions.

Among non-genetic inheritance mechanisms, parental effects represent a major determinant in the offspring phenotype and phenotypic plasticity

(Laland et al., 2015; Moore et al., 2019; Pigliucci, 2007). In the case of parental effects (see 1.2), the offspring phenotype is not only a direct response to its genetic background and prevailing environmental conditions (and their interaction), but is also affected by the environment and characteristics of its parents (a phenomenon also referred to as *intergenerational plasticity*, i.e. phenotypic plasticity that occurs from the parents to the offspring) (Bell & Hellmann, 2019; Laland et al., 2015; Yin et al., 2019; Zhang et al., 2020).

1.1.3 Adaptive versus passive phenotypic plasticity

From an evolutionary perspective, phenotypic plasticity is expected to be mostly adaptive as it allows an adjustment of the phenotype to extend tolerance to the environment toward an optimum phenotype - which is thought to ultimately increase the individual fitness and favour its contribution to the next generation (i.e. long-lasting effects). Yet, the adaptivity of phenotypic plasticity has been largely debated as empirical studies have demonstrated negative costs linked to phenotypic plasticity (Callahan et al., 2008; DeWitt et al., 1998; Via et al., 1995). Expressing phenotypic plasticity does not necessarily mean expressing the optimum phenotype, and in some cases phenotypic plasticity leads to the expression of a phenotype associated with lower fitness compared to a fixed phenotype (maladaptive plasticity) (DeWitt et al., 1998; Ghalambor et al., 2007; Pelster & Burggren, 2018). A mismatch between the organism phenotype and its environment can happen whether the phenotype is fixed or plastic (DeWitt et al., 1998). When environmental conditions are outside the range of optimal conditions for the organism, it may create *environmental stress* (Ghalambor et al., 2007). In this case, phenotypic plasticity can occur as an *adaptive response* to mitigate the effects of environmental stressors (e.g. change in resource allocation), or occur as a *passive response* where phenotypic plasticity is a consequence of environmental stressors (e.g. resources limitation impacting the phenotype) (Van Kleunen & Fischer, 2005).

Assessing the adaptivity of phenotypic plasticity is of high interest in evolution, but is laborious task as i) testing the impact of a phenotype on the

individual fitness can be challenging (e.g. wild species), ii) the advantages of a phenotype are context-dependant, and iii) transient costs can be associated with expressing a different phenotype (Chevin & Hoffmann, 2017; Metcalfe, 2024). Beside assessing the potential adaptivity of a phenotype, the mechanisms underlying plasticity in developmental trajectories remain poorly understood, and further research is needed to explore the consequences of variation in development at a cellular level (e.g. costs linked to different development, such as molecular damages) (Metcalfe, 2024; Westneat et al., 2019).

1.2 Parental effects

1.2.1 Non-genetic inheritance: different forms of parental effects

Several mechanisms enable the transmission of environmental cues from parents to the offspring, including epigenetic processes, the transmission of developmental resources (e.g. nutrients, hormones), micro and small-interfering RNA transfer, transmission of microbiome, or behavioural interactions (e.g. parental care) (Bell & Hellmann, 2019; Jablonka & Raz, 2009; Sengupta et al., 2023).

Numerous studies have investigated the mechanisms and conditions underlying intergenerational plasticity (and parental effects). In marine sticklebacks (*Gasterosteus aculeatus*), the offsprings reached larger sizes (a fitness related trait) when their rearing environment was similar to their maternal environment, and such differences were partly explained by a modulation of the offspring metabolism (Shama et al., 2014). Interestingly, the grandmother environment modulated the grand-kids metabolism as well (*transgenerational plasticity*, i.e. phenotypic plasticity that occurs across generations), with higher mitochondrial metabolism for the individuals originated from mother developed at higher temperatures (Shama et al., 2016). A study carried out in Orange-fin anemone fish (*Amphiprion chrysopterus*) demonstrated that parents living in high water flow environment produced offsprings with a different caudal fin shape (compared to parents living in low water flow), which may help the

offspring to maintain their position in high water flow environment (Cortese et al., 2022). Despite a higher mortality, such morphological changes for the surviving fishes may reflect adaptive plasticity in response to the parental environment.

Parental effects, however, do not necessarily lead to adaptive response (Bonduriansky & Crean, 2018; Burgess & Marshall, 2014; Marshall & Uller, 2007; Sánchez-Tójar et al., 2020; Uller et al., 2013; Yin et al., 2019). For instance, gestating female rats exposed to pesticides produced offsprings (F1) and grand-offsprings (F2) with pathological phenotype (e.g. testis and ovarian diseases). Such transgenerational effects could be explained by the fetal exposure of F1 to pesticide, and subsequent germ cell exposure (F2) (Manikkam et al., 2012).

1.2.2 Maternal hormonal effects

The transfer of hormones from the mother to the offspring represents an important mediator of maternal effects (in fishes: Faught & Vijayan, 2018; in birds: Groothuis et al., 2005, 2019; Ruuskanen & Hsu, 2018; in mammals: Thayer et al., 2018). Unlike in mammals, maternal-mediated hormones cannot be continuously provided to the offspring in avian species, but are deposited into eggs before the embryo development (Groothuis et al., 2005). After egg laying, hormonal levels cannot be adjusted and should support the offspring development until its endocrine system is fully developed (Darras, 2019; McNabb, 2006; Schwabl, 1999). The hormonal deposition in eggs had been shown to depend on the mother's environment conditions, such as food availability and predation risk (Saino et al., 2005) – making birds an interesting model to investigate parental effects and their potential consequences on the offspring (Groothuis et al., 2005, 2019).

While the effects of maternal androgens have been extensively studied (Groothuis et al., 2005; Podmokła et al., 2018), thyroid hormones (TH) remain understudied, despite their role on the offspring early development of phenotype (Ruuskanen & Hsu, 2018). TH have been shown to affect many aspects of the offspring phenotype, including the offspring behaviour and early-life imprinting (in fishes and precocial birds) (Bett et al., 2016;

Yamaguchi et al., 2012), but also offspring growth through the modulation of growth factors (e.g. insulin, glucagon, catecholamines), and downstream regulatory hormones (Grøntved et al., 2015; Pucci et al., 2000; Sinha et al., 2018).

Glucocorticoids (GCs) represents another class of key maternal hormones (Sheriff & Love, 2013). GCs have been shown to promote developmental plasticity in many tissues (e.g. brain, liver, cardiovascular system, determination of the Hypothalamic-Pituitary-Adrenal axis in humans) (Seckl, 2004), and in many species (Thayer et al., 2018) - notably through the modulation of genome expression (Le et al., 2005; Xavier et al., 2016). GCs are mostly studied in regards to their involvement in *stress response* (Bebus et al., 2020; Crespi et al., 2013; Love & Williams, 2008). However, GC baseline levels are also expected to influence offspring traits due to their key role different metabolic processes, and to vary between individuals according to age, food restriction, predation risk, conspecific density and individual quality (Angelier et al., 2010; Guindre-Parker, 2020; Jenkins et al., 2014; Schoech et al., 2011). In avian species, experimental elevation of GCs (e.g. corticosterone) in eggs has been shown to modify offspring behaviour, with higher begging rates for nestlings exposed to corticosterone (in European starlings *Sturnus vulgaris*, and yellow-legged gull *Larus michahellis*) (Love & Williams, 2008; Rubolini et al., 2005). During postnatal development, GC oral supplementation reduced growth rates in zebra finch nestlings (*Taeniopygia guttata*) (Spencer & Verhulst, 2007). Similar results have been obtained in barn swallows, with nestlings hatched from corticosterone supplemented eggs having lower a body mass, and smaller tarsus length and rectrix (Saino et al., 2005).

However, results of studies investigating the effect of prenatal hormonal levels on offspring traits are contrasting, and the consequences on offspring survival and fitness (or fitness-related traits) can be difficult to assess (Groothuis et al., 2020). The mechanisms underlying variation in offspring traits are unclear, and further research is needed to comprehensively understand the impact of prenatal hormonal levels on offspring development.

1.2.3 Postnatal parental investment: offspring provisioning

Beside influencing the prenatal (e.g. *in ovo*) environment, parental effects can also modulate postnatal developmental conditions, such as nutritional provisioning (Giordano et al., 2014; Uller, 2008). In birds, the number of chicks in the nest (i.e. brood size) is often used as a proxy of postnatal rearing conditions as it is expected to be associated with sibling competition (Bebbington et al., 2017; Neuenschwander et al., 2003), early-life nutritional conditions (Bebbington et al., 2017), nest temperature (Andreasson et al., 2016; Arct et al., 2022) and stress (e.g. parasitism:Badás et al., 2023). Brood size manipulations (i.e. enlarging or reducing the brood size) have been extensively used to assess the impact of the number of nestlings in the nest and associated parental care on the offspring traits. At first glance, larger clutches seem beneficial for the parents as a higher number of nestlings (if successfully fledged) should lead to a higher number of offspring recruited, and therefore a higher fitness. However, large clutches require more food and parental effort than small ones, sometimes leading to a decrease in nestling body mass and size at fledgling if the postnatal provisioning does not meet the offspring requirements during the growth period (Gosler, 1993). In Great tits (*Parus major*), individuals raised in enlarged broods have lower body mass and size at fledging (a fitness-related trait), and lower chances to be recaptured a few months after fledging (Hõrak, 2003; Rytkönen & Orell, 2001; Smith et al., 1989; Tinbergen & Boerlijst, 1990). Similar results have been found in Zebra finches: nutritional deficit (one possible consequence of being raised in large broods) impacted the laying initiation, hatching success, antioxidant markers, and flight performance on a longer-term (Blount et al., 2006; Criscuolo et al., 2011). Prior research demonstrated that telomere length (in interaction with parasitic pressure in jackdaws, *Corvus monedula:* Badás et al., 2023) and oxidative stress (in Eurasian kestrel, *Falco tinnunculus:* Costantini et al., 2006*;* in European starlings in interaction with low-quality year: Bourgeon et al., 2011) can decrease concomitant with the brood size. Yet, the physiological and molecular mechanisms underlying (i) offspring phenotype in response to early-life conditions, but also (ii) long-term effects of these early-life conditions (e.g. notably on survival and fitness) remain poorly understood (Metcalfe, 2024).

1.3 Variation in metabolism and impact on phenotypic plasticity

1.3.1 Variation in whole-organism metabolic rate

Among plastic traits, variation in metabolic rate is one important candidate pathway driving the plastic response for the whole organism, as it is directly involved both in energy allocation processes (determining growth trajectories), and in individual fitness (Brown et al., 2018; Burger et al., 2019, 2021; Norin & Metcalfe, 2019). Even when assessed through standardized measurements of oxygen uptakes, such as basal metabolic rate (i.e. BMR: minimal rate of oxygen consumption at resting at thermoneutrality) or resting metabolic rate (i.e. RMR: minimal rate of oxygen consumption at resting), oxygen uptake varies within species and is likely to have consequences on individual performance and fitness (Burton et al., 2011; Norin & Metcalfe, 2019; Pettersen et al., 2018; Speakman et al., 2004). Beside intrinsic characteristics of the individual, such as body mass, sex, diet, fasting status, reproductive and migratory states, immune system function, extrinsic factors (e.g. developmental temperature, early-life nutrition, social stimuli) also seems to create variation between individuals (review in Burton et al., 2011; McKechnie, 2008).

Metabolic rate (e.g. RMR) is also modulated by environmental factors, in response to ambient temperature (Anttila et al., 2013; Briga & Verhulst, 2017; Broggi et al., 2007; Williams & Tieleman, 2000), and seasonality (review in McKechnie, 2008; Swanson, 2010). Whereas some early-life environmental factors can be difficult to take into account in studies, the brood size can be used as a proxy of the offspring early-life environmental conditions in birds. Interestingly, brood size has been shown to influence offspring metabolism in zebra finches in the long-term, with individuals raised in large broods displaying higher metabolic rate later in life (1-year old) compared to individuals raised in small broods (Verhulst et al., 2006).

While RMR seems to have some genetic basis (Nafstad et al., 2023; Nilsson et al., 2009; Rønning et al., 2007; Sadowska et al., 2005), offspring RMR can also be modulated by parental effects (Pettersen et al., 2024), including

maternal effects through the transfer of hormones into eggs (Burton et al., 2011). Among maternal hormones, GCs act as central regulators of metabolism homeostasis and are involved in many metabolic process, including the regulation of glycogenolysis and lipolysis (Rose et al., 2010; Sapolsky et al., 2000). Early-life exposure of zebra finch nestlings to GCs (i.e. oral corticosterone treatment during postnatal development) has been shown to increase their metabolism (Spencer & Verhulst, 2008). Other studies also demonstrated the impact of maternal glucocorticoids on the offspring cardio-metabolism (Aghajafari et al., 2002; Eberle et al., 2021). TH are another important family of hormones modulating metabolism (Pucci et al., 2000; Sinha et al., 2018). For instance, *in ovo* elevation of TH hormones increase the offspring RMR at hatching in some birds (Hsu et al., 2017), but also modulates energy expenditure in humans (Harper & Seifert, 2008; Kim, 2008).

Despite the amount of articles, reviews and opinion pieces on variation in metabolic rate, the underlying molecular mechanisms explaining this variation and its consequences for the individual phenotype and performance (survival and fitness) remains currently unclear (Burton et al., 2011; Metcalfe et al., 2023). Furthermore, measuring the whole-organism oxygen uptake does not provide precise information on energy flux and energy expenditure (Metcalfe et al., 2023), a promising avenue to explore how mechanistic constraints at a cellular-level, i.e. in mitochondria, may shape variation in metabolism and offspring phenotype (Heine & Hood, 2020; Koch et al., 2021).

1.3.2 Variation in mitochondrial metabolic rate

The conversion of resources into energy happens in mitochondria, where oxidative phosphorylation transforms metabolic fuel into readily available biochemical energy storage (ATP synthesis). The efficiency of this conversion varies within species according to the individual life stages and in response to physiological needs (Metcalfe et al., 2023; Salin et al., 2015, 2019). Conversely, the efficiency of the conversion of resources into energy is also likely to play a fundamental role in explaining individual heterogeneity in growth, reproduction and survival (Goodrich & Clark,

2023; Heine & Hood, 2020; Koch et al., 2021; Salin et al., 2019).

At first glance, a higher mitochondrial metabolism may seem beneficial, but increased ATP production also comes at a cost. Higher metabolic rate generates more pro-ageing compounds, such as reactive oxygen species (ROS), which are inherent sub-products of mitochondrial respiration. These pro-ageing compounds may damage cell composition and functions (a process known as oxidative stress) and promote cellular senescence (Dawson & Salmón, 2020; Salin et al., 2018; Sastre et al., 2003). Therefore, mitochondrial efficiency is likely fine-tuned according to the benefits and costs of producing more ATP or pro-ageing compounds at given life-history stages and within given ecological contexts. A growing body of evidence demonstrates that mitochondrial phenotype and efficiency indeed vary within species together with environmental conditions, such as cold exposure, elevated temperatures, altitude and food restriction (Chung & Schulte, 2020; Gyllenhammer et al., 2020; Le Roy et al., 2021; Mahalingam et al., 2020; Salin et al., 2019; Scott et al., 2018; Zitkovsky et al., 2021). Moreover, mitochondrial metabolic efficiency is also modulated in the process of *stress response* in several species (Manoli et al., 2007; Sokolova, 2018).

To date, much attention has been given to laboratory animal models (mostly mammals) and little knowledge is available regarding the plasticity of mitochondrial traits in response to the environment in wild species, and especially in birds (Koch et al., 2021). Indeed, the physiological mechanisms and environmental determinants underlying variation in mitochondrial metabolism and its efficiency, but also the consequences of this variation on the individual phenotype and performance are as yet underappreciated (Heine & Hood, 2020; Koch et al., 2021). For instance, it remains unclear whether variation in mitochondrial metabolism promotes plastic responses (e.g. by impacting energy allocation in growth) and if such effects are carried across life and have an impact on the “final” phenotype. Moreover, the potential benefits associated with a modulation of mitochondrial metabolism remain poorly understood.

Birds present a key advantage over mammals for the study of mitochondrial metabolism. As red blood cells (RBC) are nucleated in birds (an ancestral

trait lost in mammals) (Yap & Zhang, 2021), avian erythrocytes possess mitochondria, and have been an early research focus for metabolic studies (Isaacks et al., 1976b, 1976a), enabling less-invasive and, crucially, longitudinal sampling. The recent development of High-Resolution Respirometry (HRR) instruments has made this approach more prevalent in ecological studies, in particular when carried out in the wild (Koch et al., 2021; Stier et al., 2013). Mitochondrial metabolism measurement methods using HRR technology have thus been developed using blood in birds (Stier et al., 2013, 2015, 2017), and such methods increased the interest for the use of fresh RBC to measure mitochondrial metabolism in avian species, notably in passerines such as Coal tits (*Periparus ater*), Blue tits (*Cyanistes caeruleus*), Great tits (Nord et al., 2021), Pied Flycatchers (*Ficedula hypoleuca*) (Stier et al., 2019); but also in domestic species such as Zebra finches (Dawson & Salmón, 2020) or in more exotic species such as King penguins (*Aptenodytes patagonicus*) (Bourguignon et al., 2017; Monternier et al., 2014; Stier et al., 2017).

1.4 Aims of the thesis

The purpose of my thesis is to establish how environmental conditions and stressors experienced by wild birds in their natural environment during the breeding period may drive the inter-individual variation observed in mitochondrial traits. In addition, I aim to investigate the consequences of early-life variation in mitochondrial traits on offspring phenotype, including morphology (body mass and size), growth trajectory, and survival.

Through four chapters, I investigate the potential determinants (including stressful situations) of a plastic response in RBC mitochondrial metabolism. In particular, I explore the contribution of non-genetic inheritance and maternal effects (here, through prenatal hormonal deposition in eggs, **Chapter I**), the effects of early-life food abundance (through sibling competition, **Chapter II**), the impacts of fasting periods experienced by the parents during the breeding season (during egg incubation, **Chapter III**), and the environmental differences experienced by the offspring across the

breeding season according to the timing of breeding (early vs. late-born chicks, **Chapter IV**).

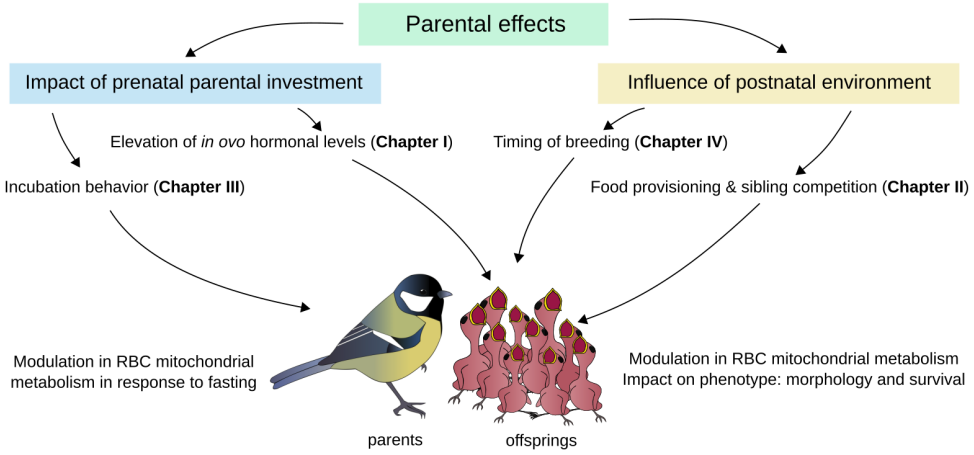

Fig.1: Summary of the aims of the thesis

2. Material and Methods

2.1. Model species

My doctoral research focuses on two distinct avian species presenting contrasting life histories and adaptations to their environment, which allow me to address different research questions by benefitting from the specific advantages that each model species provides. I first deployed an experimental approach (**Chapters I and II**) in the Finnish population of great tits (*Parus major* Linnaeus 1758) on Ruissalo Island in Turku, Finland (60°N). The usage of artificial nest boxes by the great tits during the breeding season and the rapid growth of nestlings in large broods offered the opportunity to both manipulate prenatal exposure to maternal hormones, but also to investigate the impact of the brood size on nestling phenotype.

Then, through a collaboration with the French Polar Institute (IPEV), I had the opportunity to use a natural study system based on the king penguin population (*Aptenodytes patagonicus* Pennant 1768) in Crozet Archipelago in the French Southern Territories (46°S) (**Chapters III and IV**). Both king penguin breeders and their single chick rely on long fasting periods in the breeding cycle, which allowed me to investigate the impact of food deprivation on RBC mitochondrial metabolism on both the parents (Chapter III) and the offspring (Chapter IV). This population represents an ideal model to investigate the impact of natural fasting and has been studied for more than 30 years. Moreover, the hatching date is a known predictor of early-life adversity in king penguins, with early-born chicks faring considerably better than late-born chicks. Studying the king penguin

enabled me to assess the impact of early-life adversity on growth and RBC mitochondrial metabolism in a study system where the adversity of rearing conditions naturally varies between chicks.

2.1.1 The Great tit model

2.1.1.1 Ecology of the Great tit

The Great tit belongs to the *Paridae* family and is the largest of the tits found in Finland, ranking as *Least Concern* in the IUCN red-list 2024. The Great tit is a widespread species across Europe, but also Middle East and Asia. The Great tit is generally a resident species, which appreciates deciduous or mixed forests, forest edges but also gardens. It usually feeds on insects, seed and nuts but adapts its diet during the breeding season (see below). This species is a typical short-lived income breeder. In the beginning of the breeding season, the female great tit will build a nest composed of moss and hair. The completion of the nest usually takes more than a week (Gosler, 1993). In Southwest Finland, the egg laying period starts on average in the beginning of May and lasts until mid-July, depending on the early spring temperature accumulation (Ahola et al., 2009; McCleery & Perrins, 1998). Once the female starts laying, she usually lays one egg per day until the completion of the clutch, with a variation in clutch size going from 7 to 12 eggs (Gosler, 1993; Perrins & McCleery, 1989). Due to their large clutch size, great tits have been extensively used as a model species to conduct brood size manipulation (Hõrak, 2003; Rytkönen & Orell, 2001; Smith et al., 1989). The female usually starts the incubation (between 12 and 15 days) before the completion of the clutch, creating hatching asynchrony between nestlings within a nest (Boles et al., 2007). During the nestling stage, which lasts from 18 to 21 days, both parents provide food to the chicks in the nest - a diet mostly composed of lepidopteran caterpillars (60 to 95% of the nestling diet, according to the availability in the environment) (Gosler, 1993; Perrins & McCleery, 1989; Royama, 1970).

2.1.1.2 Study area and study system

In **Chapters I and II**, the research was carried out in Southwest Finland,

on Ruissalo Island in Turku, Finland (60°26.055′N, 22°10.391′E). The University of Turku disposes artificial breeding nest boxes (n = 588 nest boxes in total) dispersed throughout the island, and suitable for the Great tit, providing an excellent system to deploy an experimental approach in the wild.

As previous data on this population demonstrated that great tits dispersion was limited in this area (Ruuskanen et al., unpublished data), specific mist-netting sessions have been organized all over the island a few weeks after each experiment (autumns 2019 and 2020, **Chapters I and II**) to increase the chances to recapture individuals that participated in the experiments as nestling. Mist-netting sessions are also organized all year around in the nearby Ruissalo Botanical Garden, which also provides supplementary information on potential recapture during the whole year.

2.1.2 The King penguin model

2.1.2.1 Ecology of the King penguin

The King penguin species belongs to the *Sphenicidae* family and is the second largest penguin species (after Emperor penguins, *Aptenodytes forsteri*), ranking as *Least Concern* in the IUCN Red List 2024. The habitat of the King penguin encompasses Subantarctic waters and the Southern Ocean. The King penguin is a land-dependent marine species, which exclusively feeds in the open sea. It relies on terrestrial environments too, mostly subantarctic islands for moulting and breeding, which imposes fasting periods.

Due to their peculiar ecology, king penguins have been the focus of ecological and physiological research for decades, notably for the physiological challenges they encounter during foraging or during their fasting periods on land.

The King penguin's breeding cycle as a long-lived capital breeder involves extended fasting periods, both for the parents (up to 5 weeks for the males) and the offspring (up to 5 months for the chick) (Cherel et al., 1987, 1988a; Cherel & Le Maho, 1985; Stonehouse, 1960). After the completion of the pre-breeding moult, king penguins return to sea to forage, and then come

back to the colony to start the courtship period. After mating and securing a spot in the colony, the female lays a single egg and returns at sea, while the male starts the egg incubation by extending the fasting period that has already started during courtship (Stonehouse, 1960; Weimerskirch et al., 1992). Parents will then alternate between foraging periods at sea and fasting periods in the colony to provide parental care (e.g. incubation, territory defence, protection from predators), with fasting shifts becoming progressively shorter as the austral summer and breeding season advance (Stonehouse, 1956; Weimerskirch et al., 1992). During the breeding season, king penguins specialize their diet towards myctophid fishes (up to 98% of the diet) and squids during the egg incubation and the chick provisioning period (Cherel et al., 2002; Cherel & Ridoux, 1992). During their foraging trips, king penguins travel quickly to the polar front where they can benefit from a higher concentration of prey stock (Bost et al., 2009).

Hatching occurs after ca 54 days of incubation. The single chick remains under the brood patch of the parent until it reaches its thermal independence around one month after hatching (Barré, 1978; Duchamp et al., 2002). When independent, the chick can be left unattended in the colony and will be regularly fed by the parents. The number of feeding events decreases as the breeding season advances, until the chick starts an almost complete winter fast (starting on average in April for the population in Crozet) (Weimerskirch et al., 1992). A large proportion of chicks die from starvation, cold or predation (Weimerskirch et al., 1992). The surviving chicks are refed at the end of winter, then moult and depart at sea in the early spring or during the austral summer, ca 11 months after hatching (Ancel et al., 2013; Stonehouse, 1960; Weimerskirch et al., 1992). The extremely long rearing period of the chick is a particularity that makes the king penguin breeding cycle quite unique among birds (Stonehouse, 1956, 1960; Weimerskirch et al., 1992). When their chick has successfully fledged (departed at sea), king penguin breeders still need to complete their breeding moult. As a consequence, they cannot start the following breeding cycle on time (Stonehouse, 1956, 1960; Weimerskirch et al., 1992). Because of this time-delay, the onset of laying can be described as biphasic, leading to early hatching around January (early-born king penguin chicks) and late hatching around February-March (late-born king penguin chicks).

Interestingly, late breeders are not necessarily successful breeders from the previous season, but also include young birds with little experience (Olsson, 1996; Stephen Dobson et al., 2008; Stonehouse, 1960).

2.1.2.2 Study area and study system

The research work conducted on king penguins was carried out on the Possession Island in Crozet Archipelago (46°25′S; 51°52′E), in collaboration with the program IPEV #119 ECONERGY from the French Polar Institute. Data were collected during the breeding season 2020-2021 (**Chapter IV**), and 2021-2022 (**Chapters III and IV**) in the “Baie du Marin” colony, which gathers around 20,000 breeding pairs (Barbraud et al., 2020). Breeding pairs that participated in these studies were randomly selected in the border of the colony during courtship.

2.2 Data collection

2.2.1 Experimental manipulation

2.2.1.1 Impact of prenatal parental investment: elevation of *in ovo* hormonal levels

The level of deposition of *in ovo* hormones from the mother to the offspring varies according to environmental conditions, and is expected to promote offspring developmental plasticity (Darras, 2019; Groothuis et al., 2019; Ruuskanen & Hsu, 2018). Yet, little information is known regarding the modulation of RBC mitochondrial metabolism by maternal effects and prenatal hormonal levels. The aim of **Chapter I** was to mimic an increase in maternal hormone levels deposited into eggs within physiological range (2SD of hormonal content measured in the study population, as recommended by Podmokła et al., 2018) and to investigate the impact of such prenatal effects on great tit chick RBC mitochondrial metabolism, growth and survival. Because of their roles on growth and developmental process, but also their impact on metabolism (see introduction), we focused this experimental study on thyroid hormones (TH) and glucocorticoids (here corticosterone, CORT).

Before the onset of incubation, all great tit eggs within a nest were randomly supplemented with the same treatment: either with thyroid hormones (TH: a mixture of 0.325ng T4 and 0.041ng T3 per yolk), corticosterone (CORT: 0.202ng per yolk), a mix of thyroid hormones and corticosterone (CORT + TH: 0.325ng of T4 + 0.041ng of T3 + 0.202ng of CORT), or a control solution (CO: an injection of control isotonic saline solution, 2µl NaCl), leading to 4 experimental groups (Fig.2). Body mass (measured with electronic scale ±0.1g) and body size (wing length measured with metal ruler ±1mm) were recorded during the growth period (days 2, 7 and 14) to assess the impact of prenatal hormonal elevation on nestling growth. Blood samples (~30–75 µl from the brachial vein using heparinized capillaries) were collected on days 7 and 14 to measure mitochondrial density and metabolism (see methods below and sample-sizes in Fig.2). Fledging success and nestling recruitment as juveniles (9 to 10 weeks after fledging) and as adults (15 to 18 months after fledging)

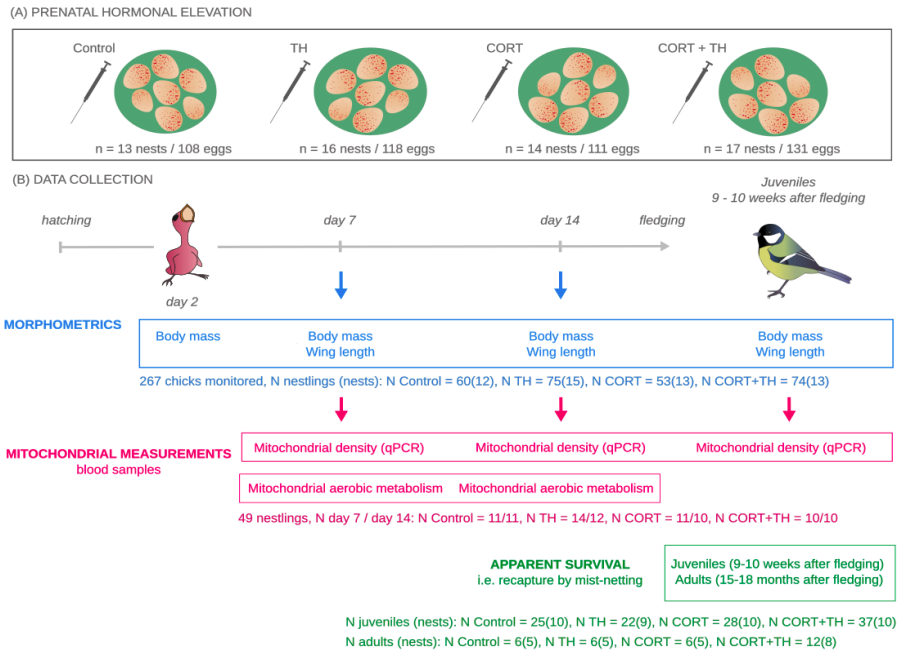

Fig.2: Experimental design of the study presented in **Chapter I** with (A) Prenatal hormone elevation and (B) data collection. Nest sample-sizes are presented according to treatment groups. The prenatal treatment led to 4 groups: control (saline solution), thyroid hormones elevation (TH), corticosterone elevation (CORT), thyroid hormones and corticosterone elevation (CORT + TH). Sample-sizes by treatment groups are presented.

were recorded to assess the impact of the prenatal hormonal supplementation on the offspring's survival (Fig.2). When recaptured, we measured body mass and size of the individuals from the experiment.

2.2.1.2 Influence of postnatal environment: brood size manipulation

The number of nestlings in the nest (brood size) has been shown to influence sibling competition and growth trajectories in great tits, with individuals raised in larger broods usually reaching lower body mass and size at fledging (a fitness-related trait). The purpose of **Chapter II** was to investigate whether brood size could influence great tit chick RBC mitochondrial metabolism during the growth period as well. Variation in RBC mitochondrial metabolism according to the brood size could explain the discrepancies observed in body mass and size between chicks raised in small or large broods. In this study, we conducted a brood size manipulation experiment, combined with a cross-fostering experiment (Fig.3). Nest box occupancy by the great tits was monitored and we recorded laying and hatching date (±24h) for each nest. Nests with equal hatching date (nest-pairs) were selected for the study. The brood size manipulation was carried out 2 days after hatching, where half of the brood was cross-fostered between nest-pairs. The objective of the cross-fostering was to assess the contribution of the original nest (representing the genetic background, prenatal and early postnatal parental effects) *versus* the contribution of nest of rearing (representing the postnatal environment from 2 days post-hatching to fledging) in nestling RBC mitochondrial metabolism. The brood size manipulation led to 3 treatment groups: (1) a control group (C, i.e. half of the brood was cross-fostered but no modification of the brood size), (2) a reduced group (R, i.e. half of the brood was cross-fostered and the brood size was reduced by 2 individuals moved to the nest-pairs), (3) an enlarged group (E, i.e. half of the brood was cross-fostered and the brood size was enlarged by 2 individuals coming from the nest-pairs) (Fig.3).

Parental feeding rate was video-recorded 8 days post-hatching on a subsample of nests (n_C = 8, n_E = 15, n_R = 14 nests) to assess whether postnatal food provisioning differed between treatment groups. During the

growth period, nestling body mass and size were recorded on days 7 and 14 post-hatching (see sample-sizes in Fig.3). Blood samples were collected on day 2 to measure mitochondrial density, and on days 7 and 14 to measure mitochondrial density, RBC mitochondrial metabolism and oxidative stress status (ROS measurements). Unfortunately, blood quantity collected on 2 days-old nestlings was too low (1–10µl) to measure mitochondrial metabolism. Recapture probability was used as a proxy of survival. Juvenile body mass and size were recorded and blood samples collected to measure mitochondrial density and metabolism.

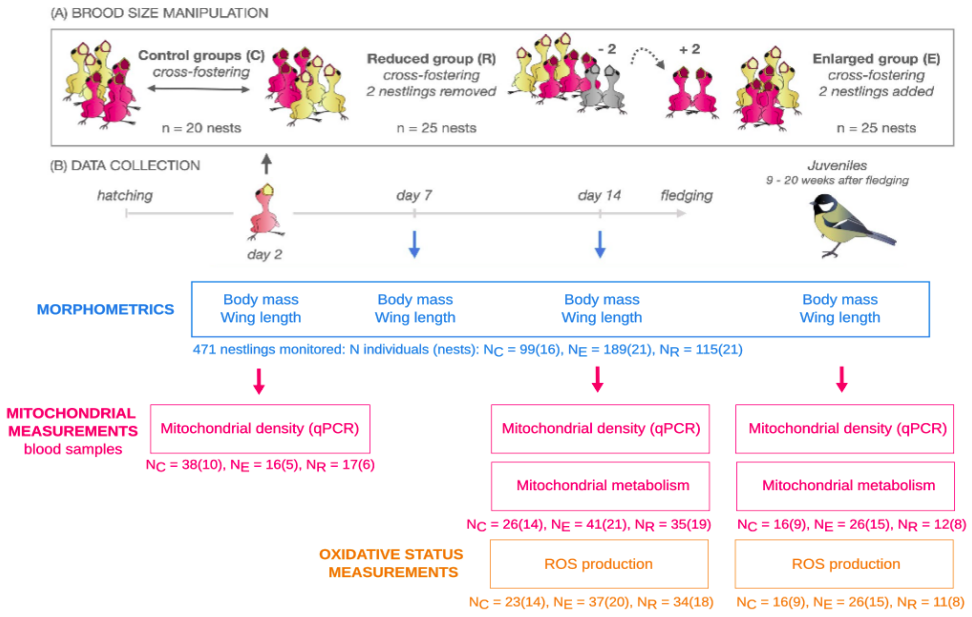

Fig.3: Experimental design of the study presented in **Chapter II**. (A) Brood size manipulation. (B) Data collection. Sample sizes (in number of nests) are presented for each treatment group: control (C), reduced (R) and enlarged (E) broods. Figure adapted from Cossin-Sevrin et al. (2023)

2.2.2 Observational studies

2.2.2.1 Impact of prenatal parental investment: incubation behaviour

Both female and male king penguins experience extended fasting periods during the breeding cycle. Breeding fasts represent a major interest in eco-evolutionary biology as their success influences the survival of both the breeders and their offspring - and therefore the breeder's fitness. The ability to fast and to cope with extended periods without food resources is a requirement for the survival of the offspring. The different phases of fasting and the associated metabolic processes have been well described in breeding king penguins (Cherel et al., 1988a; Groscolas et al., 2008; Groscolas & Robin, 2001), yet the efficiency of energy synthesis from metabolic resources at cellular level is poorly studied. The aim of **Chapter III** was to investigate how natural fasting experienced by both female and

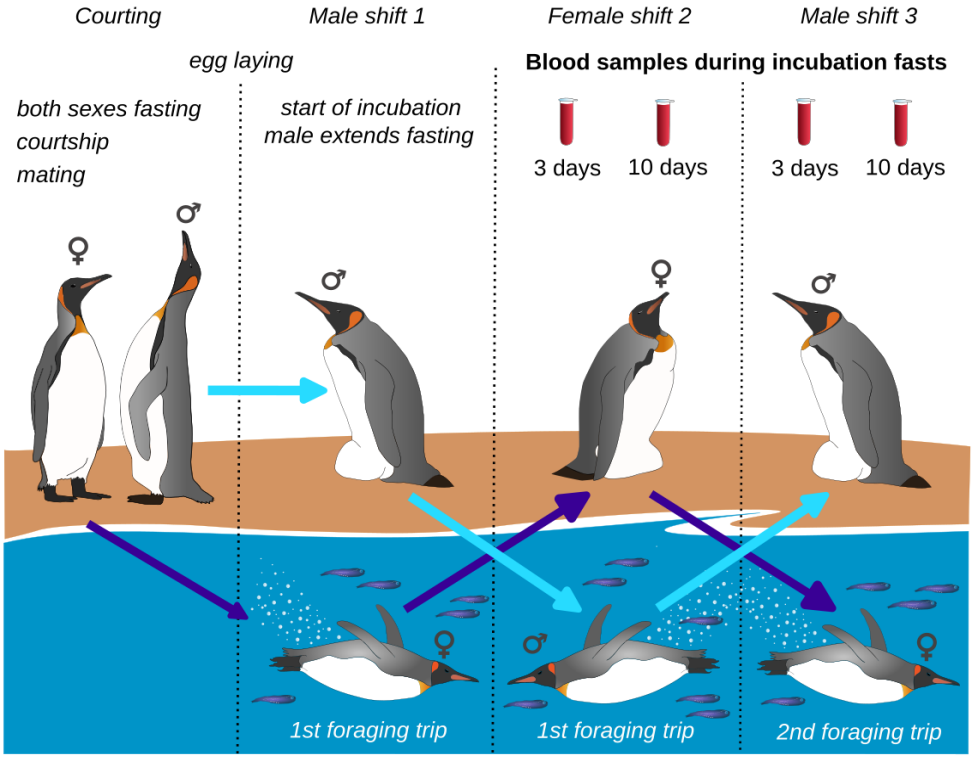

Fig.4: Schema of the king penguin breeding cycle and design of the study presented in **Chapter III** (i.e. start of the egg incubation period)**.** Figure adapted from Chapter III.

male king penguins during their breeding cycle modulates their RBC mitochondrial metabolic rates and metabolism efficiency. By focusing on the parental side in this Chapter III, I aimed to investigate how the ability of the parents to cope with fasting (at the cellular level) could possibly impact the incubation behaviour (and potentially parental care). Beside testing if RBC mitochondrial metabolism is modulated by the extended fast, I also investigated whether such modulation would differ between sexes. For this study, 40 breeding pairs were monitored during the egg-incubation period (measurements: n_{day3} = 70 and n_{day10} = 65 from 40 females and 39 males). In order to measure RBC mitochondrial metabolism, blood samples were collected in the beginning of the fasting period (3 days after arriving on land) and at the end of the fasting period (10 days after arriving on land). To draw similar comparisons between sexes, we collected blood samples in females during their first shift and males during their second shift to ensure data were collected at similar stages of fasting: both sexes came back from their first foraging trip after the egg laying to resume incubation (Fig.4).

2.2.2.2 Impact of prenatal parental investment: timing of breeding

For all king penguin chicks, the growth period (ca 11 months) encompasses different stages, including a period of rapid body mass increase during the first summer (hereafter referred to as the *core growth period*), followed by a period of limited food provisioning (Cherel et al., 1987; Cherel & Le Maho, 1985; Stonehouse, 1956, 1960). Natural phenotypic differences exist between early- and late-born king penguin chicks. Late-chicks are usually smaller and lighter than early-ones and possess less body reserves when entering into winter. They are therefore facing a much higher mortality than early-chicks (Fernandes, 2023; Stier et al., 2014; VanHeezik et al., 1993). Whereas the variation in morphometrics between early- and late-chicks have been demonstrated in several studies, little is known regarding potential differences in metabolism. The aim of **Chapter IV** was to test if RBC mitochondrial metabolism differ between early- and late-chicks and to assess whether different growth trajectories and distinct phenotypes could be linked to and predicted by a variation in RBC mitochondrial metabolism. To this end, we monitored early- and late-chicks during two breeding seasons (2020-2021 and 2021-2022). Morphometrics and RBC

mitochondrial respiration were measured at different stages across the whole growth period: namely, during the core growth period (35 days posthatching), at the end of the core growth period (100 days posthatching) and during winter (150 days posthatching) (see timelines and sample-sizes presented in Fig.5). The objectives were to test whether RBC mitochondrial metabolism would differ during the period of rapid body mass gain, but also when chicks are facing challenging conditions during winter, when parental provisioning is limited.

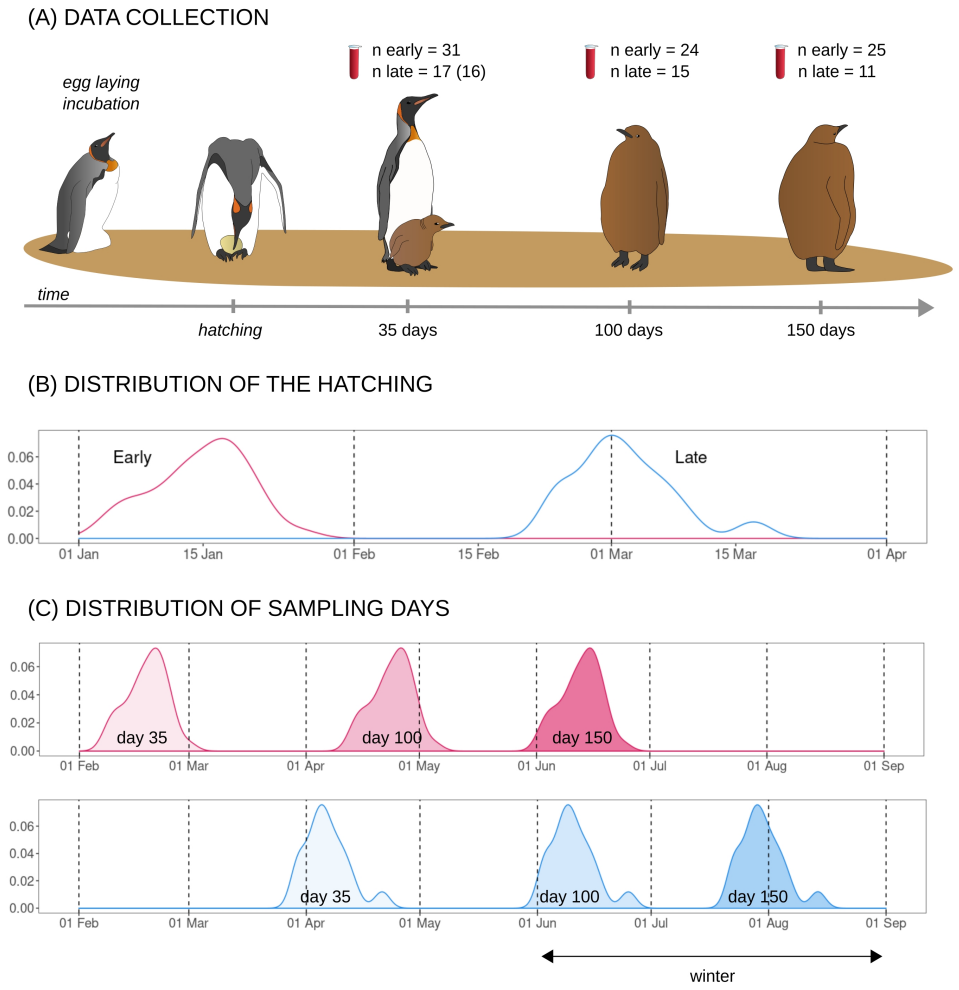

Fig.5: Schema of the study design presented in **Chapter IV**. Representation of the king penguin breeding cycle (i.e. egg laying to the beginning of the rearing period) and data collection of morphometrics and mitochondrial metabolism measurements (A), density distribution of hatching dates (B), and density distribution of sampling days (C). Sample-sizes for mitochondrial respiration measurements are presented. A total of 55 individuals were included in this study: N(early) = 31 and N(late) = 23 individuals. For day 35 measurements, one value was missing for *ROUTINE* (sample size in brackets). Figure adapted from **Chapter IV.**

2.2.3 Mitochondrial aerobic metabolism

2.2.3.1 In vitro respirometry using the Oroboros platform

In vitro mitochondrial aerobic respiration was measured using the *Oroboros* Instrument, which is composed of two respirometry chambers (O2k-chamber) and a polarographic oxygen sensor. The polarographic oxygen sensor measures the concentration of dissolved dioxygen in the liquid - a respiration buffer with the biological sample, loaded into the O2k-chamber. Once the O2k-chamber is closed, the concentration of dioxygen declines over time as a result of the mitochondrial respiration by the sample. By using a substrate-uncoupler-inhibitor titration (SUIT) protocol (see SUIT lists and actions in Fig.6 below), and measuring the subsequent oxygen concentration decline in the O2k-chamber, we can assess the oxygen flux (JO_2, i.e. the time derivative of the decline in oxygen concentration: $\Delta[O_2]/\Delta t$) and calculate the catabolic flux issued from the sample (i.e. the time derivative of the decline in oxygen concentration corrected for instrument background flux and the non-mitochondrial respiration).

2.2.3.2 In vitro respirometry using permeabilized red blood cells

The protocol for HRR was adapted for each species in this thesis. To simulate the body temperature when measuring mitochondrial metabolism in vitro, the temperature of the O2k-chamber was set at 40°C for the great tits and 38°C for the king penguins (Stier et al., 2017). Our protocol allowed us to measure six different respiration rates.

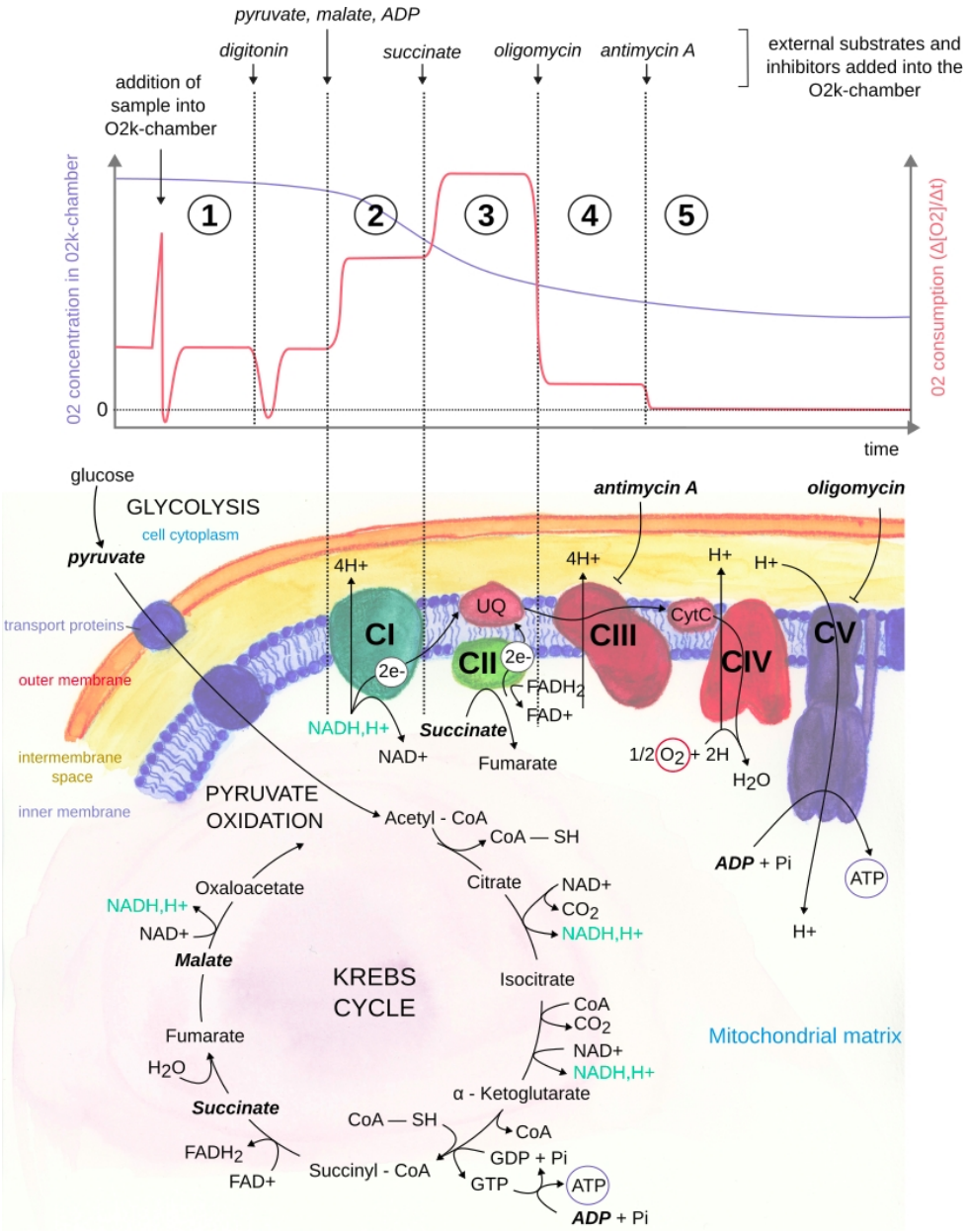

Fig.6: Schema of the SUIT protocol for HRR. On top, change in O2 concentration (purple curve) and flux (red curve) as measured in the O2k-chamber. The different steps of the SUIT protocol are indicated with numbers (from 1 to 5) – see the matching numbers in the main text for the description of each step. At the bottom, mitochondrial electron transport system with its different elements are represented. **CI:** mitochondrial complex I (NADH dehydrogenase), **CII:** complex II (succinate dehydrogenase), **CIII:** complex III (ubiquinol-cytochrome C oxidoreductase), **CIV:** complex IV (cytochrome c oxidase), and **CV:** ATP synthase. **UQ:** Ubiquinone and **CytC:** cytochrome C.

(1) After loading the sample (20-50µL RBC, depending on the model species, diluted into mitochondrial respiration buffer MiR05 respiration buffer), closing the O2k-chamber and waiting for stabilisation of the signal, we first measured the endogenous respiration of intact cells (i.e. oxygen consumption in the sample before cell permeabilisation and before the addition of exogenous substrates and inhibitors, ***ROUTINE***, step 1 on the Fig.6). *ROUTINE* represents the general metabolic activity of the living cells under the experimental conditions in the O2k-chamber (e.g. fixed temperature). *ROUTINE* depends on physiological activity in the sample, but also on the availability of intra-cellular metabolic substrates and ADP levels (Gnaiger, 2020).

(2) *Digitonin* (20µg/mL), an agent that permeabilises cell membranes, is then added in O2k-chamber. After stabilisation (*ca* 5 min), exogenous substrates of the complex I: *pyruvate* (5mM), *malate* (2mM) as well as ADP (1.25mM) are added in the O2k-chamber. O2 consumption increases and reflects the maximal respiration rate of the complex I (***CI***, step 2).

(3) Mitochondrial complexes I and II work in tandem to sustain the oxidative phosphorylation. After measuring CI, we then add the exogenous substrate of the complex II: *succinate* (10mM), to measure the maximal respiration capacity provided by both complexes I and II (***CI+II***, step 3).

(4) To estimate the non-phosphorylating respiration incurred through proton leak (***LEAK***, step 4), *oligomycin* (2.5µM), an inhibitor of the ATP synthase (sometimes also called mitochondrial complex V) is added in the O2k-chamber (Devenish et al., 2000). Following the addition of *oligomycin*, O2 consumption decreases and reflects the consumption linked to uncoupled respiration (respiration not linked to ATP synthesis).

(5) Finally, to measure the non-mitochondrial (e.g. contaminant bacterial) respiration, *antimycin A* (2.5µM) an inhibitor of the reduction of cytochrome C (by binding to the Qi oxidation site in CIII), is added in the O2k-chamber, and entirely blocks all mitochondrial respiration (Step 5, decrease in O2 consumption). Non-mitochondrial respiration is considered constant, and subtracted from all respiration rates. Rates used in this thesis are always corrected for non-mitochondrial O2 flux: for instance, *ROUTINE* refers to initial oxygen consumption as measured in Step 1,

from which non-mitochondrial respiration is subtracted (Gnaiger, 2020).

Several indirect respiration rates are then calculated. *Complex II respiration* (***CII***) refers to the maximal respiration rate from the mitochondrial complex II alone and is calculated as *CI+II - CI*. ***OXPHOS*** refers to the respiration rate supporting the ATP synthesis through oxidative phosphorylation and is calculated as *CI+II - LEAK*.

Three mitochondrial flux control ratios (FCR) are also calculated: (1) *OXPHOS* coupling efficiency (***OxCE***), which refers to the mitochondrial metabolism efficiency to synthesize ATP in relation with the maximal respiration capacity and proton leak. *OXPHOS* coupling efficiency is calculated as *OxCE = (CI+II – LEAK)/(CI+II)*. At the individual level, a high value of *OXPHOS* coupling efficiency means that the part of the respiration dedicated to ATP synthesis (*OXPHOS*, coupled respiration) is large in comparison to LEAK (the uncoupled respiration). In a classical system where ATP is the main form of energy for cellular processes, this is therefore a more efficient metabolism (a higher conversion rate from nutrients to energy). Naturally, metabolic efficiency may be defined differently in a tissue where the desired product of metabolism is heat, and not ATP (e.g. in brown adipose tissue; Nedergaard & Cannon, 2018). We also calculated (2) **FCR *ROUTINE/CI+CII*** represents the proportion of the maximal respiration capacity being used under the endogenous cellular respiration rate, and (3) **FCR *CI/CI+II*** is the ratio between complex I maximal respiration rate and the maximal respiration capacity. As FCR are ratios, they are independent from cell count, sample volume and mitochondrial content (Gnaiger, 2020).

2.2.4 Standardization of the mitochondrial metabolism measurements

For **Chapters I and II**, metabolic rates were standardized by the RBC count of each sample measured before conducting HRR, using a Bio-Rad TC20 automated cell counter. Unfortunately, for **Chapters III and IV** blood samples from king penguins could not be standardized by cell count as we detected a huge variability in cell count from the instrument used in

the field, and this could not be changed due to the remoteness of the research station. We therefore quantified the total amount of proteins in the samples recovered from the O2k-chamber as a proxy for the cell count, using the Pierce™ BCA Protein Assay Kit (assays performed on frozen samples, kept at -20°C) instead. Mitochondrial metabolic rates were either divided by the cell count, or by the total amount of proteins measured in samples.

2.2.5 Mitochondrial density and oxidative status

In **Chapters I and II**, mitochondrial DNA copy number (i.e. mtDNAcn, a proxy for mitochondrial density) was measured in great tit blood samples, using real-time quantitative Polymerase Chain Reaction (qPCR). qPCR analysis allows the detection and quantification of targeted DNA molecules in a given sample (G. Adams, 2020). Cytochrome oxidase subunit 2 (COI2) was used as a specific mitochondrial gene (as it is normally not duplicated in the nuclear genome) and Recombination Activating Gene 1 (RAG1) was used as a reference (single copy gene present in the nuclear genome). Both DNA sequences are amplified and quantified using fluorescent DNA-binding dyes (see details of qPCR primers and reaction steps in original publications). The ratio between COI2 and RAG1 provide an estimation of the relative mtDNA copy number for each sample. To conduct qPCR analysis, genomic DNA was first extracted from 1-5µl of frozen blood (stored at -80°C) using a salt extraction procedure (adapted from Aljanabi & Martinez, 1997). DNA quantity and purity were estimated using spectrophotometry with a NanoDrop ND-1000 spectrophotometer, and sample DNA was re-extracted if needed (see details in the original publications). DNA integrity was evaluated using gel electrophoresis and samples were diluted and stored at -80°C until qPCR analyses (see more details in the original publications).

In **Chapter II**, reactive oxygen species (i.e. ROS) were measured in 14 days-old great tit nestlings and juveniles in samples recovered from the O2k-chamber (RBC suspended in MiR05 buffer). To this end, the relative amount of ROS was estimated by fluorescence, using a MitoSOX™ Red kit (MitoSOX™ red mitochondrial superoxide indicator, Thermo Fisher).

This kit measures the primary mitochondrial ROS (i.e. mitochondrial superoxide) in living cells (see more details in the original publication).

2.3 Data analysis

All statistical analyses were performed using R software v.4.0.2 (http://www.R-project.org/) and using the generalised linear mixed-effects models (GLMMs) or linear mixed-effects models (LMMs) from *lme4* package (Bates et al., 2015). The main statistical tests conducted in my thesis are presented below (more details can be found in the original publications and manuscripts). For all analyses, criteria of normality was assessed with a Cullen and Frey plot from *fitdistrplus* package (Delignette-Muller & Dutang, 2015). *emmeans* package was used to conduct multiple *posthoc* comparisons tests (with Tukey honest significance differences – HSD – correction). Standardized effect sizes (Cohen's d) were estimated using *effsize* package (Torchiano, 2020). Values were considered as statistically significant when $P < 0.05$.

In **Chapter I**, to test the effect of prenatal hormonal treatment on great tit nestlings growth, mitochondrial density, metabolism and survival, I used LMMs and included the CORT treatment (2-levels: yes/no) and TH treatment (2-levels: yes/no), hatching date (continous variable), day 2 brood size (continuous variable) as fixed effects and nest ID as random intercept. The interaction between CORT and TH treatments was preliminarily tested, but removed from the models if non-significant in order to properly interpret the treatments as main effects. To analyse great tit nestling growth trajectories (body mass across age), age was added as fixed factor (3-levels: day2/ day7/ day14) and bird ID as random intercept. Great tit nestling body size (i.e. wing length) and body condition (scaled mass index as calculated in Peig & Green, 2009) were analysed at each age with nest ID included as random intercept. As mtDNAcn did not fulfil the criteria of normality, I used GLMM with a gamma error distribution (log link) to evaluate treatment effect (CORT and TH included as fixed factors) on mitochondrial density across time (age included as fixed factors, 2-levels: day7/day14), with nest ID and bird ID included as random intercepts. Mitochondrial metabolic rates were analysed (LMM) both at a

cellular level (mitochondrial metabolic rates included as response variables), and at a mitochondrial level (mitochondrial metabolic rates included as response variables, corrected for mitochondrial density, i.e. including mtDNAcn as a covariate in the models).

In **Chapter II**, great tit nestling growth trajectories, mitochondrial density, metabolism, ROS production and survival were tested by including the brood size manipulation treatment (3-levels: Reduced, Control, Enlarged), hatching date (continuous variable) and initial brood size before treatment (continuous variable) to account for different initial brood size between nests. Both the original nest ID and nest of rearing ID were included as random intercepts in the models. The interaction between brood size manipulation treatment and initial brood size was always tested, and removed from the model if non-significant. For mtDNAcn (GLMM with gamma error distribution) and body mass (LMM), age was included in the model as a fixed factor (3-levels: day2/day7/day14) and bird ID as a random intercept. The interaction between age and treatment was initially tested but removed from the model if non-significant. Again, mitochondrial metabolic rates were analysed (LMM) both a cellular- and mitochondrial-level (with mtDNAcn included as covariate). In this Chapter, I also used another statistical approach (correlative approach) by including the actual number of nestling in the nest on the day of data collection as continuous variable in the models (instead of including the treatment and initial brood size). The contribution of the variance explained by the original nest and the nest of rearing was estimated using *RptR* package (gaussian distribution, N bootstraps = 1000) (Stoffel et al., 2017).

For both **Chapters I and II**, survival metrics (hatching success, fledging success and recapture probability) were analysed using a logistic GLMM with a binomial distribution of the survival outcome.

In **Chapter III**, because mitochondrial metabolic rates and FCR did not fulfill the criteria of normality, these response variables were analysed using GLMMs with gamma error distributions (log link). To test if mitochondrial metabolic rates differed between king penguin breeder sexes and fasting duration, sex (2-levels: female/male) and fasting duration (2-levels: day3/day10 of fasting) were included as fixed effects and the bird

ID as a random intercept. The interaction between sex and fasting duration was preliminarily tested, and later removed from models if non-significant.

In **Chapter IV**, king penguin chick body mass, size and condition, as well as mitochondrial metabolic rates (except *LEAK*) were tested using LMM against the age (3-levels: day35/day100/day150), the chick group (2-levels: early/late), the year of sampling (2-levels: 2020/2021) as fixed factors and the bird ID as a random intercept. *LEAK* was analysed using a GLMM with gamma error distribution (log link). To assess differences in overall metabolic profiles between early and late-chicks, I performed a linear discriminant analysis (LDA) and included *ROUTINE*, *CI*, *CI+II* and *LEAK* (other indirect metabolic rates could not be included as they are already linear combinations of the others). To test if growth trajectories could be predicted by mitochondrial metabolism, I tested differences in body mass between day 35 and day 100 (body mass gain during the core growth period), but also the mass differences between day 100 and day 150 (changes in body mass during winter), using LMM with mitochondrial metabolic rates measured at day 35 and day 100 (or day 100 and day 150 for winter mass differences) both included as continuous variables, and the chick group (early/late) and year of sampling (2020/2021) as fixed effects.

3. Main Results and Discussion

A recurring theme throughout my thesis was the modulation of offspring RBC mitochondrial metabolic traits by its prenatal and postnatal environments, and the impact of this modulation on the offspring's phenotype, including body mass and size, but also survival. The main results of my thesis are summarized below and complementary details can be found in the original publications.

3.1 The timing of breeding can create adverse early-life conditions (Chapters I, II, IV)

In the chapters that focused on the offspring (**Chapters I, II, IV**), I investigated the contribution of the hatching date in chick RBC mitochondrial metabolism, growth trajectories and survival. Whereas the **Chapter IV** has been specifically designed to investigate the effect of the hatching date on the offspring phenotype, **Chapters I and II** also provide relevant insights.

Interestingly, during the breeding season 2019-2020 (**Chapter I**), great tit nestling body mass and size significantly decreased with the hatching date (predicted body mass loss: -0.28g per week delay, roughly -8,5% of 2 days-old great tit chick mass raw data average). Such differences in body mass and size according to the hatching date were not visible any more at the juvenile stage, where individuals reached their final body size. Independently from the prenatal hormonal treatment, the respiration capacity of the complex I (*CI*), the maximal respiration capacity (*CI+II*), the proportion of respiration linked to oxidative phosphorylation (*OXPHOS*) and mitochondrial metabolism efficiency (*OXPHOS* coupling efficiency)

slightly but significantly increased with the hatching date. As the endogenous respiration (*ROUTINE*) did not vary across the season, the ratio between the endogenous respiration and the maximal respiration capacity (FCR *ROUTINE/CI+II*) decreased with the hatching date. In this case, great tit chicks hatched late in the season had lower body masses and smaller sizes, but these differences were not visible in the long run (in juveniles). Here, it is possible that higher transient RBC metabolic rates in late-hatched nestlings supported the energy expenditure linked to a faster compensating growth rate, as they reached a similar size as individuals hatched earlier in the season. Despite non-optimum conditions, late-chicks thus managed to compensate for a deficit in body mass and size during the growth period.

However, breeding early is not always an advantage. For the breeding season 2020-2021 (**Chapter II**), I surprisingly found opposite results. Great tit nestling body mass and size increased strongly with the hatching date (predicted body mass gain: 0.63g per week, ca +21.4% of 2 days-old great tit chick mass raw data average), meaning that early-hatched chicks were smaller and lighter. Unlike Chapter I, *CI*, *CI+II*, *OXPHOS*, *OXPHOS* coupling efficiency significantly decreased with the hatching date. Due to the decrease in *CI+II*, FCR *ROUTINE/CI+II* increased with the hatching date. However, *ROUTINE* did not vary across the season (as in Chapter I). Here as well, early-hatched great tit chicks manage to compensate for a deficit in body mass and size during the growth period, probably with an increase in RBC mitochondrial metabolism and efficiency during the growth period. Again differences in morphormetrics were not visible anymore in juveniles.

Whereas the causality link between the growth trajectories and RBC mitochondrial metabolism is difficult to assess (see results below for Chapter IV), both studies provide evidence on the importance of the timing of breeding in the great tit chicks phenotype and mitochondrial metabolism profile. Numerous studies aimed at understanding the relationship between breeding phenology, reproductive performance and fitness (Both et al., 2006; Gilsenan et al., 2020; Shipley et al., 2020; Verhulst & Nilsson, 2008). The timing of breeding depends on environmental cues, such as photoperiod, food peak availability, ambient temperature, but also on the

breeders' experience and body condition (Helm et al., 2013; Marrot et al., 2018; Nilsson & Källander, 2006; Perrins, 1970; Visser et al., 1998). In some cases, the environmental cues influencing the timing can be more difficult to interpret (especially in the context of global changes) (Visser et al., 2004, 2021), and may increase the likelihood of mistimed reproduction (i.e. reproduction occurring in non favourable conditions, for instance too early or too late) (Both et al., 2006; Visser et al., 1998). Mistimed reproduction has been shown to have drastic consequences on species and populations (Both et al., 2006). The results presented above support the fact that the hatching date should not be interpreted by itself (as similar hatching dates can be associated positively or negatively with the offspring traits), but should be interpreted in balance with the environmental conditions to estimate whether the reproduction was mistimed or not (Vatka et al., 2014).

In case of the king penguin, the position of Antarctic Polar Front is a main determinant of breeding success - and of mistimed reproduction. During winter, the Antarctic Polar Front and its high concentration of myctophid fishes (main prey of king penguins during breeding) is shifting southward, and therefore extending the foraging trips for the parents (Bost et al., 2004; Cherel & Ridoux, 1992; Freeman et al., 2016; Jouventin et al., 1994). The withdrawal of the Antarctic Polar Front during winter thus imposes long periods of fasting to the chicks that remain in the colony and cannot yet feed by themselves (Cherel et al., 1987; Cherel & Le Maho, 1985). Winter thus imposes harsh conditions on the king penguin chicks, and even more so for the late-born ones as they usually start winter with lower body reserves and face a higher mortality during winter (Fernandes, 2023; Stier et al., 2014; VanHeezik et al., 1993).

The aim of **Chapter IV** was to study the differences in king penguin chicks phenotype according to their hatching date (early *versus* late-chicks), and in particular to test for potential differences in RBC mitochondrial traits between the two groups of chicks. I also investigated if differences in body mass and size across the whole growth period, which includes a core growth period with fast body mass gain (from day 35 to day 100) and a period of fasting during winter (from day 100 to 150), could be predicted by the chicks RBC mitochondrial metabolism and its efficiency. Here, my results

were consistent with the literature (Fernandes, 2023; Stier et al., 2014; VanHeezik et al., 1993): late-chicks differed from early-chicks' phenotype at the end of the core growth period and onwards, with lower body mass (-40.3% at day 100), lower body size (-22.3% at day 100) and lower body condition (-16.5% at day 100). Late-chicks reached the crèche stage (i.e. the collective huddling of chicks unguarded by their parents) at younger age compared to early-chicks (respectively average ± s.e.m: 31.00 ± 0.86 *versus* 35.06 ± 0.34 posthatching days). Consistent with our expectation, late-chicks had a drastically reduced fledging success compared to early-chicks (34.4% of survival for early-chicks fledging *versus* 8.7% for late-chicks), most probably because they did not acquire enough body fat and protein reserves to cope with the reduction in parental feeding rates during winter (Cherel et al., 1987; Fernandes, 2023; Stier et al., 2014; VanHeezik et al., 1993; Verrier, 2003). However, the survival metrics presented here represented a biased sample, as we only monitored individuals that survived at least until 35 days posthatching (i.e. with some selective disappearance) - but similar results have been found using larger and less biased samples (Fernandes, 2023).

Interestingly, I showed that late-chicks expressed a higher RBC mitochondrial metabolic profile at the end of the core growth period (day 100) compared to early-chicks. Late-chicks had a higher endogenous respiration (*ROUTINE,* +48.8%), but the maximal respiration capacity (*CI+II,* measured after cell permeabilisation with external substrates) did not differ between chick groups. As a consequence, the ratio between the endogenous respiration and the maximal respiration capacity (FCR *ROUTINE/CI+II*) increased as well (+33.7%). In other words, late-chicks consumed more oxygen and used a higher proportion of their maximal respiration capacity, without increasing the part of the respiration dedicated to ATP synthesis (similar *OXPHOS* between chick groups) and without increasing mitochondrial metabolic efficiency (no differences observed in *OXPHOS* coupling efficiency). The respiration of the complex I (*CI*) was higher in late-chicks at all ages (+14.1%).

Several hypotheses could explain these differences in metabolic profiles between early and late-chicks:

(1) First, when comparing similar ages (100 days), late-chicks express higher RBC metabolic rates. Yet, early-chicks reached 100 days-old during the autumn while late-chicks reached 100 days during winter (Fig.5). Thus, higher RBC metabolic traits in late-chicks may be the consequence of facing different environmental conditions and may reflect the stress associated with different severities in food restriction (Blas, 2015).

(2) Second, as RBC mitochondrial metabolism has been shown to decrease during the growth period in relation to the reduction of mitochondrial content per cell with age (see 3.4, **Chapter I and II**, Stier et al., 2022), it is also possible that late-chicks were at a less-advanced developmental stage, and therefore had a higher mitochondrial density leading to higher mitochondrial metabolic rates compared to early-chicks. This hypothesis would be supported by the smaller body mass and size of the late-chicks. Moreover, a prior study showed that late-chicks can experience faster growth rate compared to early-chicks at the beginning of the core growth period (Stier et al., 2014), and rapid growth rates has been shown to increase mitochondrial density in other bird species (Japanese quails, *Coturnix japonica*) (Jimenez et al., 2014). This hypothesis would also be supported by the fact that the late-chicks are probably still growing at 100 days posthatching (as suggested by Fig.7, with an increase in body mass between day 100 and 150, although differences between days were non-significant).

(3) Finally, since parental provisioning is reduced during winter, variations in RBC mitochondrial metabolic rates may reflect different fasting stages and conditions between chicks, as reported in king penguin adults (**Chapter III)** and in previous research carried out in king penguin chicks (Bourguignon et al., 2017; Monternier et al., 2014; Teulier et al., 2013). In these studies, chicks captured during the winter period (July to August, time period fitting day 150 in Chapter IV) were in *phase II* of fasting, in which resting metabolic rate is decreased (-17% compared to re-fed chicks). When entering *phase III* of fasting, metabolic fuel switches from lipids to protein catabolism and mitochondrial metabolic efficiency is increased. It is therefore possible that differences between early- and late-chicks may reflect different fasting stages and conditions between individuals (e.g. comparing a chick recently fed with a chick in *phase II* of fasting, or

comparing two chicks being respectively in *phase II* and *III* of fasting).

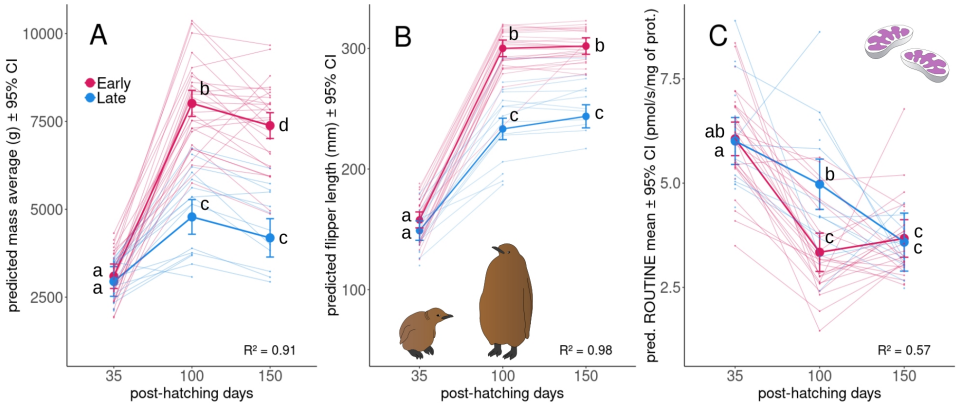

Fig.7: Predicted (thick lines) king penguin chick body mass (in g) (A), predicted body size (flipper length, in mm) (B) and predicted *ROUTINE* (pmol.s^{-1}.mg^{-1} proteins) (C) according to groups (early *versus* late-chicks) from 35 to 150 days posthatching. Predicted averages are presented with their 95% confidence interval. Letters indicate Tukey HSD *post hoc* comparisons: for each graph, if groups share a letter, they are not significantly different from each other. Raw data are plotted behind predicted average (lines). Red colour refers to the early-chicks, blue colour refers to the late-chicks. R^2 from LMMs are presented. Figure adapted from **Chapter IV**.

Overall, **Chapter IV** also provides evidence on the impact of the breeding phenology on the offspring phenotype for a particular species, which has a naturally large variance in laying date. In the present study, I did not assess the differences in parental quality between breeding pairs (such as foraging trip duration and efficiency), which have been shown to be a determinant in chick growth trajectories in king penguins (Olsson, 1996), but also in other penguin species (Marciau et al., 2023). Yet, as for king penguins the timing of breeding is associated with the success of the last breeding season, I did not expect variation in parental quality to be associated with the timing of breeding in this specific case. Yet, in many species, breeding early brings numerous advantages, such as an increase in the chances to find a suitable habitat, a good partner, and more time available for the offspring to reach their final size – all reasons explaining why selection often favour early breeding (Marrot et al., 2018; Van Noordwijk et al., 1995; Visser et al., 1998). For king penguins, breeding early may also mean avoiding metabolic stress for the offspring during the chick rearing period, which could lead to detrimental effects later in life (see 3.5).

3.2 Impact of prenatal parental investment on offspring and parents (Chapters I, III)

3.2.1 Elevation of *in ovo* hormonal levels affects the offspring mitochondrial metabolism

During the initial stages of development, the offspring relies on the transferred hormones from the mother (here into eggs) before its endocrine system is fully functional (Darras, 2019; McNabb, 2006; Schwabl, 1999). Variation in hormonal levels in eggs can have important effects on the offspring traits, including behaviour (e.g. food begging), but also physiological traits (e.g. immune system and metabolic rate) (Dufty et al., 2002; Groothuis et al., 2005; Meylan et al., 2012). In **Chapter I,** I assessed if a modification of *in ovo* environment, through an elevation of hormonal levels in eggs, would affect postnatal mitochondrial metabolism and offspring growth. As both thyroid hormones (TH) and glucocorticoids (i.e. corticosterone, CORT) are expected to modulate the offspring metabolism (Rose et al., 2010; Sinha et al., 2018; Spencer & Verhulst, 2008), and to vary according to environmental conditions (Guindre-Parker, 2020; Jenkins et al., 2014; Ruuskanen et al., 2016; Ruuskanen & Hsu, 2018), we here mimicked an increase of maternal TH and CORT by experimentally injecting great tit eggs (see Methods for more details).

While I expected nestlings hatched from TH-injected eggs to express a higher RBC mitochondrial metabolism (Cioffi et al., 2013), I did not find any evidence of such effects. The experimental elevation of TH *in ovo* did not impact the great tit chick growth pattern or mitochondrial metabolism. According to the literature, an elevation of *in ovo* TH can affect the offspring phenotype in avian species in different directions (e.g. heavier body mass 2 days posthatching, longer telomere during postnatal development, reduction of telomeres length a few days after fledging) but does not necessarily affect the offspring phenotype (Hsu et al., 2018, 2020, 2023; Ruuskanen & Hsu, 2018; Stier et al., 2020). Several hypotheses may explain these contrasting responses. Each egg already naturally contains an original amount of TH in the yolk, which is expected to vary according to temperature or food availability (Ruuskanen et al., 2016; Ruuskanen & Hsu,

2018). Therefore, the effect of elevation may be different among individuals and according to environmental conditions during pre- and postnatal development (Groothuis et al., 2020). As here TH supplementation significantly decreased nestling developmental time in eggs (-5% compared to control group injected with a saline solution), I am confident about the actual increase of TH in yolk resulting from the injection. However, TH may have impacted the measured traits only during prenatal development, or alternatively have impacted traits not measured in this study (e.g. nestling behaviour, or specific target tissues but not RBC).

According to the literature, I expected nestlings hatched from CORT-injected eggs to express higher RBC mitochondrial metabolism as well (Manoli et al., 2007), with potential costs on a longer-term (Haussmann et al., 2012; Metcalfe & Monaghan, 2001). Yet, I found opposite results. The prenatal elevation of CORT significantly reduced nestling mitochondrial density (-27.4%) a few days before fledging (day 14), leading to a decrease in most of the mitochondrial metabolic rates at a cellular-level (Fig.8). When investigating metabolic variation at the mitochondrial-level (i.e. mitochondrial respiration rates corrected for mitochondrial density), only

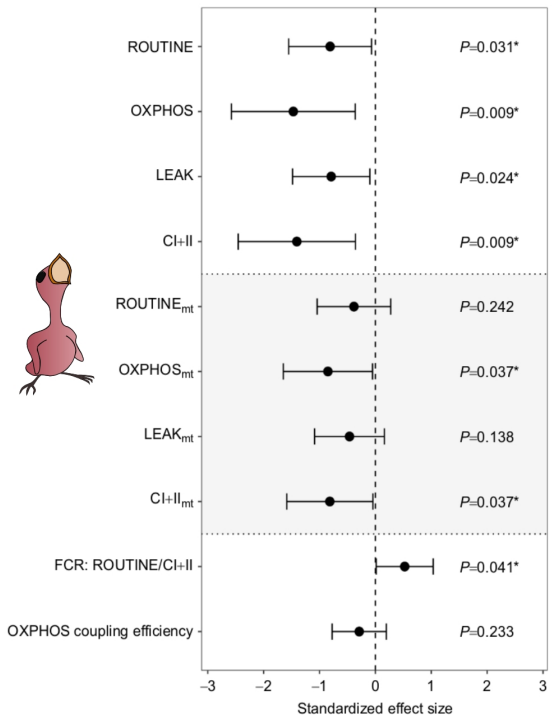

Fig.8: Effect of prenatal CORT treatment on great tit chicks mitochondrial metabolism. Here effect-sizes refer to the differences in mitochondrial metabolic rates between control chicks and chicks that received a CORT treatment. Standardized effect-sizes (with their 95% CI) are based on predicted values from LMMs. Metabolic rates with subscript mt were corrected for mitochondrial density (mtDNA copy number included as covariate in models). Measurements were made on day 7 (nCORT/non-CORT= 21/25) and day 14 posthatching (nCORT/non-CORT =20/23 individuals). The interactions between chicks age and CORT treatment were significant. Asterisks indicate significance. Figure adapted from Cossin-Sevrin et al. (2022), **Chapter I**.

the oxidative phosphorylation (*OXPHOS*) and the maximal respiration capacity (*CI+II*) remained significantly lower (respectively -14.2% and -13.3%) compared to control group (Fig.8).

Interestingly, this decrease in mitochondrial content per cell, and in metabolic rates, did not affect mitochondrial metabolism efficiency (*OXPHOS* coupling efficiency), and chicks treated with CORT most likely compensate such metabolism reduction with a higher usage of their total respiration capacity (increase in FCR *ROUTINE/CI+II*, +7.9%). As the effects of *in ovo* CORT elevation only appeared a few days before fledgling (14 days posthatching), the results of this study suggest that prenatal CORT elevation had a delayed and transient effect on the chicks during the rearing period. Despite a reduction of mitochondrial density and respiration, nestlings from the CORT-injected eggs reached on average a similar fledging body mass, size and body condition as nestlings from the control group. This absence of detectable effects on nestling morphology could be explained by a reduction of the energy requirements linked to growth by the increase of CORT, or to a change in nestling begging rate, and thus possibly in food intake, linked to an increase in CORT as reported in the literature (Rubolini et al., 2005).

Beside the delayed effect of CORT on nestlings during the growth period, I found that females recaptured later in life as juveniles (i.e. 9 to 20 weeks after fledging) had a significantly lower body mass compared to individuals recruited from the other groups. The mechanisms underlying variation in female body mass and condition later in life are unclear, but suggest that prenatal CORT treatment could have long-lasting consequences.

Whereas **Chapter I** suggests that prenatal hormonal effects can modulate the offspring RBC mitochondrial metabolism, further research would be needed to (1) assess whether an early-life variation in RBC mitochondrial metabolism leads to different metabolic profiles in a longer term (in adults), (2) unravel the mechanisms allowing prenatal hormonal levels to modulate the offspring metabolism, and (3) investigate the potential long-lasting effects of an early-life variation in RBC mitochondrial metabolism on the offspring phenotype (see 3.5).

3.2.2 Incubation behaviour impacts the parents mitochondrial metabolism

As for many other seabirds, the incubation and rearing periods of the King penguin occur on land, where the food is not available (exclusively feeding at sea), which imposes extended fasting periods (up to 5 weeks for the male king penguins) – creating physiologically challenging conditions for the parents (Cherel et al., 1988a; Secor & Carey, 2016; Stonehouse, 1960; Weimerskirch et al., 1992).

The key objectives of **Chapter III** were (1) to investigate whether the RBC mitochondrial metabolism of king penguin breeders was modulated by the duration of their incubation fast in free-living conditions, and (2) to assess whether this modulation could be sex-specific. In both sexes, the proportion of respiration allocated to ATP synthesis (*OXPHOS*) and the respiration of the complex I (*CI*) were decreased in response to fasting (-2.6% and -8.7% respectively) (Fig.9). Consequently, the ratio between respiration of the complex I and maximal respiration capacity (FCR *CI/CI+II*) also decreased at the end of fasting (-7.7%). However, most of the mitochondrial metabolic rates were not impacted by the fasting duration (Fig.9). As prior research demonstrated a reduction of oxygen consumption with fasting in king penguin males, and a decrease in resting metabolic rate (-30% on average after 15-20 days of fasting) (Fahlman et al., 2005; Rey et al., 2008), we expected an overall decrease in mitochondrial metabolism across fasting with lower rates at day 10 than at day 3. However, in this study, not all individuals displayed similar metabolic responses to fasting and we found a high inter-individual variation.

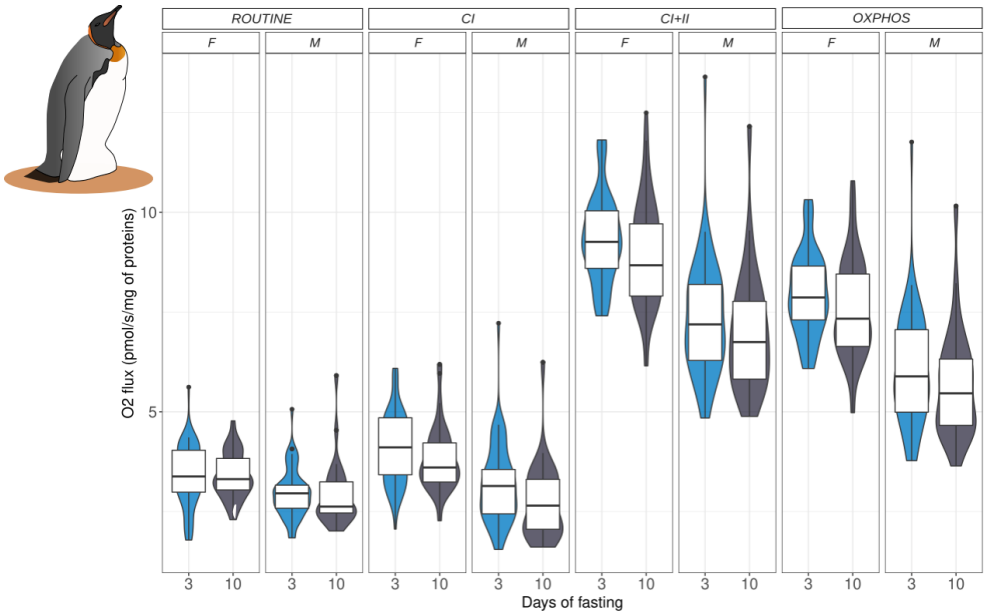

Fig.9: Differences in mitochondrial metabolic rates on days 3 and 10 of the breeding fast in female and male king penguins. Raw data distribution is shown using violin plots and encompasses 135 measurements: n(day3) = 70, n(day10) = 65 in 40 females and 39 males. Raw data are presented with boxplots. When performing LMMs, only CI and *OXPHOS* were significantly lower at day 10 compared to day 3 in both sexes. Figure adapted from **Chapter III**.

Independently of the fasting stage and the body condition, all mitochondrial metabolic rates except FCRs were higher in king penguin females compared to males (Fig.11). Unlike males for which mitochondrial metabolism efficiency (*OXPHOS* coupling efficiency) decreased with fasting (-8.7%), females were able to maintain an efficient metabolism despite lower oxidative phosphorylation (*OXPHOS*) at the end of fasting (day 10) (Fig.10). These results provide a new insight into the King penguin physiology as information on females are under-represented for this species – as it is in the field of physiology in general (Arnegard et al., 2020; Garcia-Sifuentes & Maney, 2021). Such differences in metabolic traits between sexes were surprising as the king penguin has minimal phenotypic sexual dimorphism (both sexes are almost identical physically, with approximately similar mass and size), and both sexes carry out similar tasks during egg-incubation, including territory defence, protection against predators and parental care (Kriesell et al., 2018; Stonehouse, 1960; Weimerskirch et al.,

1992). Furthermore, females and males use similar foraging areas throughout the breeding season with a low variation in their diet, even with contrasting climatic conditions (Brisson-Curadeau et al., 2023; Jouventin et al., 1994).

There is still a wide knowledge gap regarding sex-specific response of mitochondrial metabolism to fasting in birds. Yet, several hypotheses may explain differences between female and male king penguins.

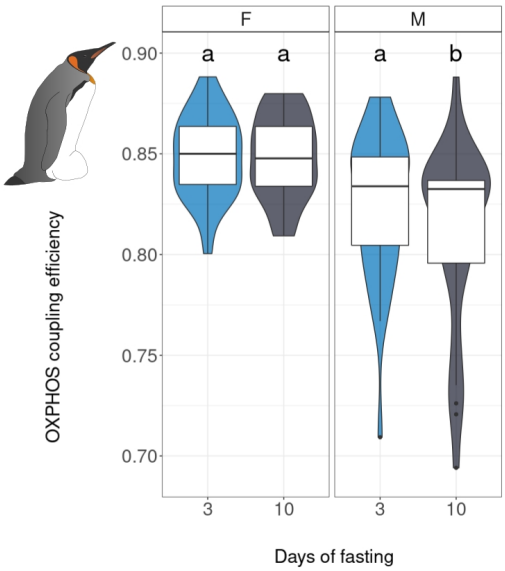

Fig.10: Differences in mitochondrial metabolism efficiency (*OXPHOS* coupling efficiency) between sexes according to fasting duration (day 3 or day 10). Raw data is shown using violin plots. The interaction between sex and fasting duration was significant. Different letters indicate differences according to Tukey HSD *post hoc* comparisons. Figure adapted from **Chapter III.**

First, our observations during king penguin breeding fasts (3 and 10 days on land) correspond to a stage of fasting (*phase II*) where lipids are used as the main resource to cover metabolic reactions (i.e. lipolysis) (Cherel et al., 1988a; Groscolas & Robin, 2001; Secor & Carey, 2016). Female king penguins may be more efficient at converting lipids into energy, as reported in short-term fasting japanese quails and in human studies in the beginning of fasting (*phase I*) and during exercise (Hedrington & Davis, 2015; Lamosová et al., 2004; Montero et al., 2018; Tarnopolsky, 2008).

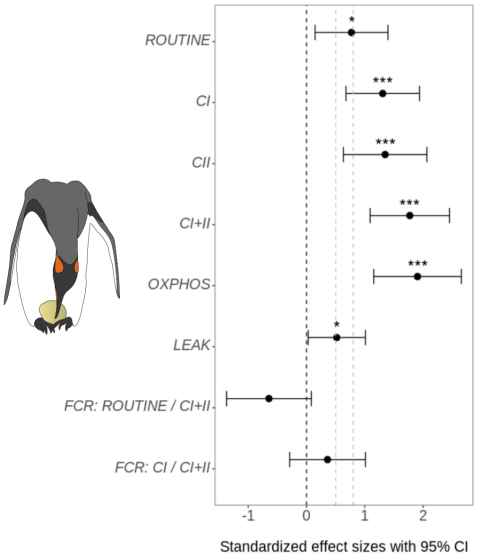

Fig.11: Differences between sexes in mitochondrial metabolic rates and flux control ratios during breeding fast in king penguins. For effect-sizes, females are compared to males (males used as a reference here). Mitochondrial metabolic rates were measured at 3 and 10 days of fasting (n day3 = 70, n day10 = 65 in 40 females and 39 males). Standardized effect-sizes (with their 95% CI) are based on predicted values from LMMs. The identity of the bird was included as a random intercept in the model to control for the non-independence of measures from the same individual. Figure adapted from **Chapter III**.

Second, recent studies (on rodents and human cells) provided evidence for the modulation of mitochondrial metabolism and its efficiency in females by the two main sex-steroid hormones (i.e. oestrogen and progesterone), by (1) preventing mitochondrial dysfunction, through receptors within mitochondria, (2) modulating the expression of ETS proteins, and (3) through receptors within mitochondria that can increase ATP production (Chen et al., 2005; Dai et al., 2013; Gaignard et al., 2018; Klinge, 2008; Price & Dai, 2015; Rosa-Caldwell & Greene, 2019; Stirone et al., 2005; Velarde, 2013, 2014). During the egg-incubation period, oestradiol levels are above the baseline in female king penguins (Jouventin & Mauget, 1996; Mauget et al., 1994). Unfortunately, only testosterone level was characterized in male king penguins (no data available for oestrogens and progesterone levels), and our study thus cannot validate the above hypothesis.

Because male king penguins experience a longer fast in the beginning of the egg incubation, prior research only focused on males to investigate the physiological adaptations that allow them to cope with prolonged fasting periods. However, females also need to meet crucial energy requirement to successfully reproduce (e.g. energy expenditure related to the egg

production and laying). As recently raised in the literature, information about females is generally missing in physiological studies (Ah-King et al., 2014; Arnegard et al., 2020; Garcia-Sifuentes & Maney, 2021; Orbach, 2022), and further research carried out on both sexes is needed to unravel potential differences in physiological adaptations between females and males.

3.3 Influence of postnatal environment on the offspring (Chapters I, II, IV)

3.3.1 Early-life stress associated with sibling competition and food provisioning

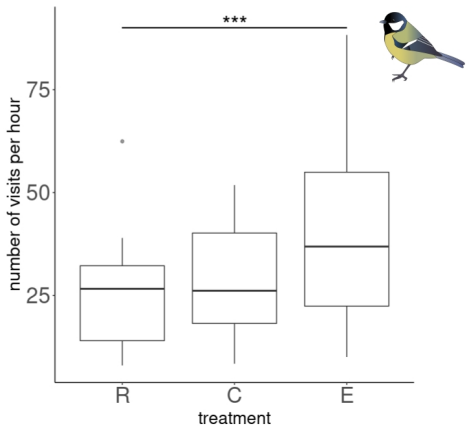

Fig.12: Great tit parental feeding rate according to brood size manipulation treatment groups: reduced (R), control (C), enlarged (E) brood sizes. Raw data distribution is presented with boxplots (n_C= 8, n_E= 15, n_R= 14 nest boxes). Stars indicate significance of Tukey HSD *post hoc* test (***P<0.001). R^2= 0.53. Figure issued from Cossin-Sevrin et al. (2023), **Chapter II**.

In the Great tit, the number of nestlings in the nest and the variation in food provisioning linked to sibling competition have been shown to influence the offspring phenotype (e.g. large brood leading to a decrease in offspring mass and size, and to a decrease in recruiting chances) (Hõrak, 2003; Rytkönen & Orell, 2001; Smith et al., 1989). Underlying mechanisms creating morphometric differences between nestlings raised in broods of different sizes could be linked to a variation in metabolic rates and energy allocation processes.

The aims of **Chapter II** were to investigate whether the brood size and subsequent variation in food provisioning associated with sibling competition would create stressful early-life conditions, leading to a modulation of the offspring mitochondrial

metabolism and its associated ROS production (proxy of oxidative stress). We also monitored nestling growth patterns to investigate potential differences in growth rates according to brood size, and recorded body mass and size a few days before fledging (a proxy of fitness in the Great tit). To this end, we conducted a brood size manipulation combined with a cross-fostering experiment (see Methods).

In this study, great tit parents managed to compensate for a higher number of chicks in the nest by increasing parental effort and postnatal provisioning (i.e. by increasing the number of visits into the nest for the enlarged group, Fig.12). Only the chicks raised in reduced broods reached a higher body mass (+4.8%) a few days before fledging but no differences were found between chicks raised in the control and enlarged broods. Moreover, the number of chicks in the nest did not remain significantly different between the control and enlarged groups at the end of the growth period. In summary, the brood size manipulation failed, in a way, to induce the planned nutritive stress for the reasons mentioned above (behavioural compensation by the parents, and non-significant differences between the control and enlarged groups), and, as a result, we did not detect any effect of the brood size manipulation treatment on nestling growth trajectories, nestling mitochondrial density, metabolism and associated ROS production, nor on juvenile traits. Therefore, I conducted complementary analyses to investigate the influence of the actual number of nestlings (rather than the treatment group) on nestling and juvenile traits.

Interestingly, the actual number of nestlings in the nest (regardless of the brood size manipulation treatment) was negatively associated with nestling mitochondrial metabolism during the growth period. Nestlings from the smallest broods (less than 5 chicks in the nest) had higher mitochondrial metabolic rates, including endogenous respiration (*ROUTINE*), maximal respiration of complex I (*CI*) and maximal respiration capacity (*CI+II*), proton leak (*LEAK*) and oxidative phosphorylation (*OXPHOS*). Such metabolic increase could be linked to a higher need for thermogenesis in nests with fewer chicks (Andreasson et al., 2018; Bicudo et al., 2001). This association did not remain significant if chicks raised in small broods (less than 5 chicks on day 14) were removed from the analyses. Two reasons may

explain this result. Higher mitochondrial metabolic rates were observed in chicks raised in broods experiencing a high mortality during the growth period, especially before day 7 posthatching (average of 1.13 nestlings lost at day 7 vs. 0.34 for larger broods). Indeed, survival chances were drastically reduced for these small broods (average on raw data: 63.4% versus 92.4% survival at day 14, excluding nests without chicks at day 14: n=12 nests). I therefore suspect that these results reflect stress in nestlings raised in suboptimal rearing conditions. An alternative hypothesis is that these individuals with higher RBC mitochondrial metabolic rates had a smaller structural size, and may be at a less-advanced developmental stage, which may explain higher metabolic rates (see 3.4). In any case it is important to keep in mind that these individuals with higher mitochondrial metabolic rates represent a non-random pool of nestlings that coped with detrimental rearing conditions in the beginning of the growth period and are subject to selective disappearance. Thus, this pattern likely represents a metabolic response linked to unfavourable rearing conditions, rather than being a general trend. However, this outcome emphasizes the importance of parental care and quality in maintaining a favourable environment for the nestlings, driving the growth and potentially the metabolism of the offsprings. We may wonder to what extent RBC mitochondrial metabolism could represent a proxy of the quality of the environmental conditions during postnatal development (see 3.5).

3.3.2 Rearing environment contributes more to offspring mitochondrial metabolism than genetic makeup

Another purpose of **Chapter II** was to estimate whether great tit nestling RBC mitochondrial metabolism was determined by the nest of origin (i.e. nest of hatching, before the cross-fostering experiment) or by the nest of rearing (i.e. nest assigned to the individual after cross-fostering experiment). The results of this cross-fostering provide evidence that not only the original nest, which represents genetic inheritance and early-life parental effects (before day 2), but also the nest of rearing that represents environmental conditions during postnatal development (from day 2 to fledging) significantly explained mitochondrial metabolic rates (except *ROUTINE* for

the nest of origin, see Fig.13).

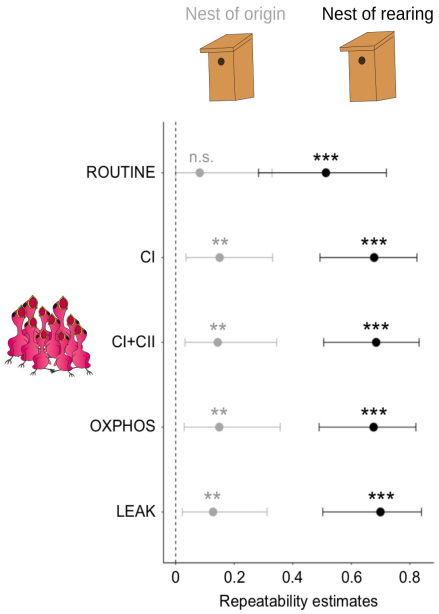

Fig.13: Variance explained by the nest of origin and the nest of rearing for each mitochondrial metabolic rate measured on 14 days-old great tit chicks. Values extracted from LMMs. Repeatability estimates are presented with their 95% CI for nest of origin (grey) and nest of rearing (black). Asterisks indicate a significant difference from 0 (***P<0.001, **P<0.01). Figure adapted from Cossin-Sevrin et al. (2023), **Chapter II.**

The nest of rearing and the environmental conditions during growth had a larger contribution in the determination of the great tit chick mitochondrial metabolism than the original nest (nest of hatching). These results are aligned with a prior study conducted on collared flycatcher (*Ficedula albicollis*), showing that resting metabolic rate was mostly determined by the environmental conditions (McFarlane et al., 2021).

A larger contribution of postnatal environmental conditions aligns with the assumption that the actual number of chicks in the nest is more important in determining the offspring growth and mitochondrial metabolism, rather than the modification of the brood size – probably because it influences the rearing conditions, in particular the temperature in the nest (Andreasson et al., 2018; Hope et al., 2021; Nord & Nilsson, 2011).

Chapter IV is also a good illustration of the importance of the rearing conditions in determining RBC mitochondrial metabolism during the growth period in king penguin chicks. Indeed, early- and late-born king penguin chicks expressed different RBC mitochondrial metabolic profiles, most likely in response to different rearing environments (see 3.1). While we cannot test the proportion of variance explained by the genetic

background *versus* the nest of rearing for this species (as there is a single chick per couple), the results of the study IV provide additional evidence supporting the influence of the rearing environment in determining chick RBC mitochondrial metabolism.

3.4 Influence of postnatal development on mitochondrial traits (Chapters I, II, IV)

3.4.1 Decrease in red blood cell mitochondrial density during postnatal development

In the great tit studies presented in **Chapters I and II**, RBC mitochondrial metabolic rates were analysed at the cellular-level (overall respiration), but also at the mitochondrial-level (i.e. respiration corrected for mitochondrial density). In both studies, mitochondrial density sharply decreased during postnatal development until it reached a minimal level a few weeks after fledging (Fig.14). Changes in mitochondrial density have been reported in the past in great tits (Hsu et al., 2023), but also in collared flycatcher nestlings during the rearing period (Stier et al., 2020).

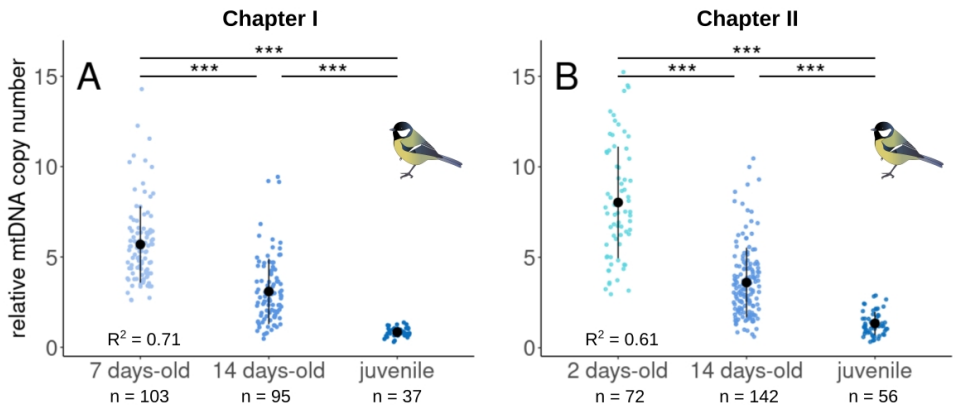

Fig.14: Changes in mitochondrial density across time in great tit chicks included in the **Chapter I** (A) and in **Chapter II** (B). Relative mtDNA copy number was measured at different time-points during the growth period and in juveniles recaptured a few weeks after fledging. Note that relative mtDNA copy number was measured on a subsample of 2 days old nestlings in Chapter II. Raw data average and SD are presented in black. Sample-sizes refers to the number of individuals in each group. Statistics are extracted from LMMs where the age was included as fixed factor and the bird ID as random intercept for Chapter I. Stars indicate significance (***, P<0.001).

The large amount of mitochondrial content per cell most likely supports the energy requirements linked to postnatal development.

3.4.2 Decrease in red blood cell mitochondrial metabolism during postnatal development

Most probably because of the decrease in mitochondrial density during postnatal development, RBC mitochondrial metabolic rates decreased during postnatal development as well, and this decrease was significant in both study species (Fig.15). In **Chapters I and II,** great tit nestling mitochondrial metabolic rates decreased until reaching a minimum level at the juvenile stage (a few weeks after fledging) (Fig.15). In king penguin chicks, RBC mitochondrial metabolic rates decreased as well, mostly between 35 and 100 days posthatching, during the core growth period. The lack of differences in RBC mitochondrial metabolic rates between 100 and 150 days-old king penguin chicks were not surprising, as chicks are reaching a body size close to their final adult size around day 100 as reported in Fig.7 (no strong differences in body size between day 100 and day 150).

There was inter-annual variation in offspring mitochondrial metabolic rates in great tits (significant differences in *ROUTINE* in 14 days-old nestlings measured in 2019 and in 2020, Fig.15). In **Chapter IV**, some mitochondrial metabolic rates were affected by the year of sampling as well (*ROUTINE, LEAK* and all metabolic parameters calculated from these two). For the study IV, the effect of the year of sampling was most likely due to a bias in our sample-sizes between years (only a few late-chicks monitored during winter of the breeding season 2021-2022). However, we cannot fully exclude that RBC mitochondrial metabolism varies between individuals according to their life-stages (e.g. postnatal development *versus* mature adult), but also according to environmental conditions as represented here by the year of the breeding season (or for instance the hatching date, see evidence presented in 3.1 and 3.3.2). Further research is needed to comprehensively interpret inter-annual variation and the context-dependent response of offspring metabolism (Koch et al., 2021).

Despite inter-annual variations in offspring mitochondrial metabolic rates, the effect of age during postnatal development on RBC mitochondrial metabolism was stronger than the variation between years (Fig.15).

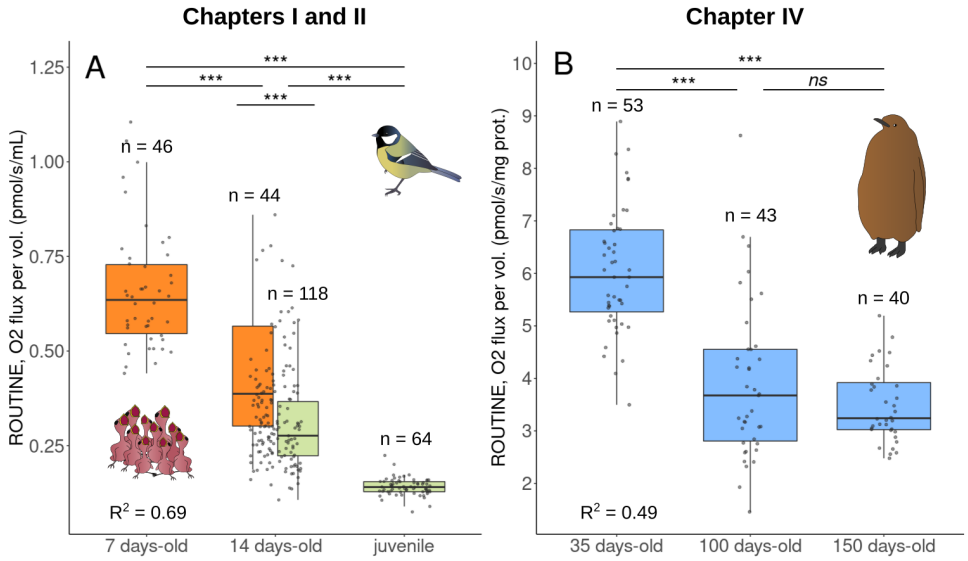

Fig.15: Changes in *ROUTINE* across time in great tit chicks included in **Chapters I** (orange colour) **and II** (green colour) (A) and in king penguin chicks included in **Chapter IV** (B). *ROUTINE* metabolic rate was measured at different time-points during the growth period (in A and B) and in juveniles recaptured a few weeks after fledging (in A, Chapter II). Raw data distributions are presented with boxplots. Statistics and R^2 are extracted from LMMs where the age of the individuals and the year of sampling were included as fixed factors. Stars indicate significance (***, P<0.001). For (B), ROUTINE from individuals of the same age did not significantly differ between years.

3.4.3 Do red blood cell mitochondrial metabolic rates predict growth patterns?

One purpose of **Chapter IV** was to test whether the offspring growth patterns could be predicted by their RBC mitochondrial metabolic rates measured at different time points during the growth period. For the king penguin chicks, I did not find a clear association between RBC mitochondrial metabolism and chick body mass gain during the core growth period, or with the changes in body mass during winter. King penguin

chicks expressing the lowest FCR *ROUTINE/CI+II* during winter (low usage of their maximal respiration capacity at day 150) were also the ones experiencing higher body mass drop during winter. This result suggests that a higher usage of the maximal respiration capacity may help the chicks to cope with the decrease in feeding events and limit the body mass drop during winter. However, the R^2 obtained from these statistical analyses were low, and results should be interpreted with caution.

I conducted similar analyses using the data collected in **Chapter I**. Unfortunately, I could not use the data collected in Chapter II as nestling RBC mitochondrial metabolism was only measured at the end of the growth period (day 14 posthatching). In Chapter I, nestling body mass gain between 7 and 14 days poshatching was not predicted by RBC mitochondrial metabolic rates, measured either at day 7 or at day 14. Once again, the R^2 obtained from these analyses were low (< 0.3).

In light of the lack of association between RBC mitochondrial metabolism and the offspring body mass gain during the growth period, and of the low R^2 obtained from the models, further studies are needed to assess whether RBC mitochondrial metabolism could indeed be a good predictor of growth trajectories.

3.5 Variation in red blood cell mitochondrial metabolic rates and potential long-lasting effects (Chapters I, II, IV)

In **Chapters I and II**, I took advantage of the study system to investigate the potential long-term effects of a variation in mitochondrial metabolic traits during postnatal development. Having productive and efficient mitochondria to increase ATP synthesis may seem beneficial for the offspring, which can meet all its requirements for postnatal development - but at the same time, increased ATP production also generates more pro-ageing products, such as ROS. When the organism cannot counterbalance the effects of pro-ageing components with an adequate antioxidant response, oxidative stress is increased, thereby promoting cellular ageing. Therefore, mitochondrial efficiency is likely fine-tuned according to the benefits and

costs of producing more ATP or ROS at given life-history stages and within a given ecological context.

In my thesis, I only measured the ROS production in individuals that participated to the **Chapter II.** I did not find any association between nestling ROS production and the brood size manipulation treatment groups. Importantly, this result was consistent with the outcomes of this study as the brood size manipulation did not impact great tit chick mitochondrial metabolism either. Individuals recaptured the following autumn did not express distinct ROS levels according to brood size manipulation treatment. However, great tit juvenile ROS levels significantly increased with mitochondrial density, suggesting that there is a trade-off between the mitochondrial content per cell, and oxidative stress.

While I did not directly measure the oxidative status of the individuals included in **Chapter I**, I found that female juveniles from the prenatal CORT supplementation had a lower body mass and body condition compared to control ones. The data collected do not allow us to answer whether long-term negative impacts came from a variation in mitochondrial metabolism during postnatal development. Furthermore, it is important to keep in mind that these results only represent the individuals recaptured in the study area. Whereas prior data suggest that the dispersion of great tits is limited in this area (Ruuskanen et al., unpublished data), our sample (both for Chapters I and II) only included individuals that survived until the juvenile stage and are recaptured, which constitutes a non-random pool. Yet, an increase in the usage of maximal respiration capacity (FCR *ROUTINE/CI+II*) coupled with a decrease in oxidative phosphorylation (*OXPHOS*) means that nestlings from the CORT group consumed more oxygen, and had stronger endogenous respiration without increasing the part of respiration linked to ATP synthesis, which overall reflects a situation where oxidative stress and subsequent cell damage may accumulate. It is therefore possible that sex-specific differences in juvenile females could be related to such metabolic alterations (Balaban et al., 2005; Costantini, 2019; Koch et al., 2021).

Whereas differences in oxidative status between king penguin early- and late-chicks were not tested in **Chapter IV**, prior literature demonstrated that

late-chicks presented higher levels of oxidative stress (D-ROM), DNA damages and telomere erosion than early-chicks (Stier et al., 2014). The authors raised the hypothesis that late-chicks accumulated oxidative stress because (1) they had higher CORT levels, (2) they were probably not able to generate an effective antioxidant response, (3) they may experience mitochondrial impairment (Stier et al., 2014). If late-born king penguin chicks indeed have consistently higher CORT levels than early-chicks, as suggest here, then this would be consistent with the results from **Chapter I**, as both great tit chicks from the CORT group and the putatively high-corticosterone, late-born king penguin chicks had a higher FCR *ROUTINE/CI+II* compared to other groups.

4. Conclusions and future perspectives

4.1 Significance of the results

Throughout this thesis, I investigated how pre- and postnatal environments, but also stressful early-life conditions, contributed to offspring RBC mitochondrial metabolism, growth trajectories and survival. I also aimed at understanding how prenatal parental investment and incubation behaviour modulate RBC mitochondrial metabolism in breeders. Taken together, the outcomes of the different chapters support the idea that environmental conditions experienced during development strongly affect the offspring RBC mitochondrial metabolism, probably more than the genetic background of the individual. However, the direction of these effects is still difficult to apprehend, and further research is needed to comprehensively understand the response of RBC mitochondrial metabolism according to environmental conditions and early-life stress. Furthermore, this thesis focuses on only a few parameters that summarise the environmental landscape of the offspring during growth, but several other parameters may need to be considered to fully assess what are the most important environmental determinants of metabolism and growth. Except in **Chapter I**, the offspring mitochondrial metabolism tended to increase in response to environmental stress (i.e. higher RBC mitochondrial metabolism for the late-born king penguin chicks, higher RBC mitochondrial metabolism for the great tit chicks growing in nest with low survival chances). In **Chapter I**, nestlings hatched from CORT-injected eggs, a proxy for a stressful environment, experienced the opposite (i.e. a decrease in mitochondrial density and metabolism, especially the part of respiration linked to oxidative phosphorylation).

However, a prior study on great tit nestlings mimicking an increase in CORT during postnatal development found different results, with an increase in proton leak leading to a decrease of RBC mitochondrial metabolic efficiency during the growth period (Casagrande et al., 2020). These contrasting results raise the hypothesis that the same hormone may have different impacts on RBC mitochondrial metabolism according to the time of exposure. Furthermore, responses to CORT have been shown to be dose-dependent (Breuner & Wingfield, 2000; Torres-Medina et al., 2018). Finally, while CORT levels can be a good indicator of stress in many species (Blas, 2015; Saino et al., 2005), increasing CORT does not necessarily mimic a situation of stress (Angelier et al., 2010; MacDougall-Shackleton et al., 2019).

Assessing whether early-life RBC mitochondrial metabolism is an indicator of an adaptive response remains challenging at this stage. First, further research is needed to assess whether individuals that express a higher (or lower) RBC mitochondrial metabolic rates early-life also express higher (or lower) RBC mitochondrial metabolism in a longer-term. This information would allow us to establish whether what we are observing is simply transient variation in metabolic traits, or actual phenotypic plasticity (Metcalfe, 2024). At first glance, higher RBC metabolic rates and the associated higher ATP synthesis should be beneficial for the organism: but the potential costs linked to higher mitochondrial respiration should be assessed both on short and longer time scales. Only thus can we positively establish whether a modulation of RBC mitochondrial metabolism is adaptive or not.

Another important question is how to define stressful conditions for the offspring. All Chapters presented in my thesis refer to parental investment either before hatching (i.e. maternal hormonal levels, incubation behaviour) or after hatching (i.e. postnatal provisioning and chick rearing). In general, parental effects are expected to improve the offspring growth and survival, as a way to increase parental fitness (Bonduriansky & Crean, 2018; Mousseau & Fox, 1998; Yin et al., 2019). But parental effects are not always adaptively matching the environmental context (Bonduriansky & Crean, 2018; Burgess & Marshall, 2014; Marshall & Uller, 2007; Sánchez-Tójar et

al., 2020; Uller, 2008; Uller et al., 2013; Yin et al., 2019). The complex association between the timing of breeding (hatching date) and chick mitochondrial metabolism in great tits (with opposite directions in different years) supports this idea. Thus, any variation in offspring RBC mitochondrial metabolism should be interpreted in balance with the individual life-stage and ecological context, in order to assess whether it is indeed the result of stress, or of a different forcing.

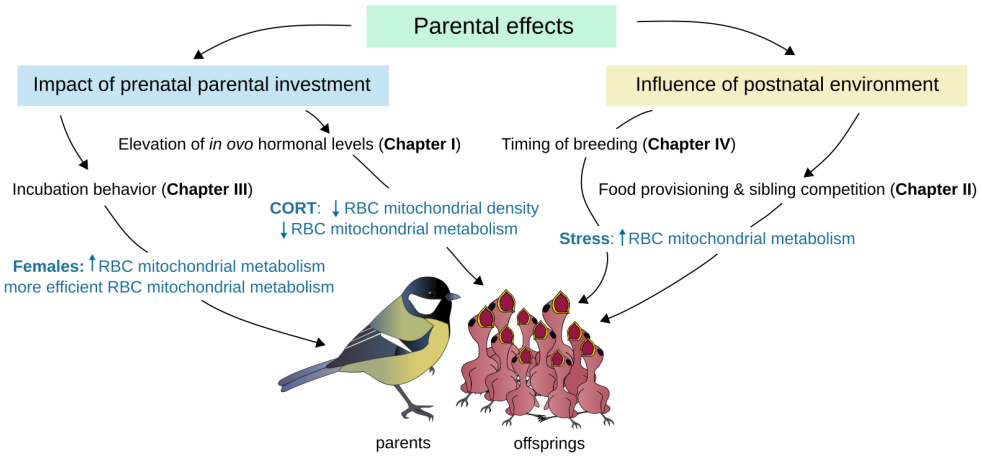

Fig. 16: Summary of the aims of the thesis and main results

4.2 Limitations of this project and future directions

Since variation in metabolic rate is central in numerous physiological and life-history traits (such as growth, immunity and reproduction), the determinants of variation in metabolic rate has been the focus of physiological researches for decades: and yet the determinants of this variation remain debated (Pettersen et al., 2018). The choice of appropriate indicators for metabolic rate (e.g. basal *versus* resting metabolic rates) is still a matter of debate, and there is a general concern about the lack of standardized methods between research works, making any comparison and conclusion between studies difficult (Pettersen et al., 2018). As the conversion into energy occurs within mitochondria at a cellular-level, mitochondrial respiration recently gained an interest in the field of ecology and evolution as it is expected to provide insights into cellular mechanisms

and constraints shaping the variation in metabolic rate (Heine & Hood, 2020; Koch et al., 2021). Yet, research on mitochondrial respiration and the usage of HRR in functional ecology is still (excitingly!) a work in progress.

(1) First, a major point of debate is the selection of a tissue to measure mitochondrial respiration. In my thesis, I exclusively used red blood cells for HRR for several reasons, including avoiding heavy procedures (e.g. terminal sampling) during sample collection, and, crucially, allowing the longitudinal collection of samples on the same individual through time. But many other tissues could be used to assess mitochondrial metabolism (e.g. whole blood, skeletal muscles, organs, isolated mitochondria). Whereas a correlation has been shown between RBC and skeletal muscle mitochondrial metabolism in king penguins (Stier et al., 2017), to the best of my knowledge, such studies are missing in great tits or other passerines. Measuring mitochondrial metabolism in other tissues may lead to different results and conclusions: further research is therefore needed to investigate how far mitochondrial metabolism correlates between tissues.

(2) Another limitation relates to the relationship between RBC mitochondrial metabolism and the whole-organism metabolic rate. In other words, can we really extrapolate our results at cellular and mitochondrial-level to the whole-organism level? And if so, to what extent? A recent study tried to assess the correlation between metabolism measured at a cellular-level and at the whole-organism levels, and, reassuringly, it appears that most of the mitochondrial metabolic rates measured on permeabilized RBC are significantly and positively associated with basal metabolic rate (Thoral et al., 2024), which greatly strengthens the results presented here.

(3) Another limit lays in the selection of the appropriate protocol to conduct HRR. Many protocols and instruments have been developed for HRR, the two most common instruments being the Oroboros Oxygraph 2k (a chamber based Clark-type electrode) and the Seahorse XF analyzer (a plate based phosphorescence system). Both have their own advantages and limitations (Walsh et al., 2023). In the four studies included in my thesis, I measured HRR using permeabilized RBC and SUIT protocol to study the response of the different mitochondrial complexes and their interactions in the intracellular environment (in opposition to isolated mitochondria or intact

cells), which yields more information about mitochondrial mechanisms (Walsh et al., 2023). Yet the usage of different protocols and the lack of standardization between studies also complicates the comparison of findings across studies, and crucial information is still missing regarding the potential impact of HRR protocols on experimental results (but see Thoral et al., 2024).

(4) An additional question relates to the standardization of the measurements and estimation of the mitochondrial content per cells. In **Chapters I and II**, I normalized mitochondrial metabolic rates by the cell count in samples. However, cell counts were difficult to obtain for the blood samples collected in king penguins (low repeatability due to technical difficulties in the field). In **Chapters III and IV**, I therefore normalized mitochondrial metabolic rates by the total content of proteins in samples – a suboptimal approach, designed to adapt to fieldwork conditions. Additionally, it has been recently shown that blood cell counts were not always associated with mitochondrial respiration (Thoral et al., 2024).

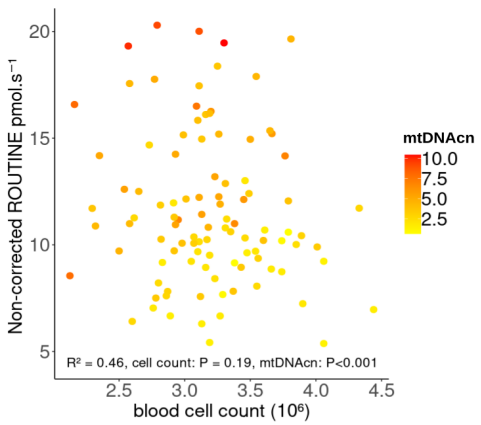

Fig.17: Association between raw *ROUTINE* (not corrected for non-mitochondrial respiration, nor for cell count, in pmol.s^{-1}) and blood cell count (10^6 cells.mL^{-1}) according to relative mitochondrial DNA copy number (mtDNAcn). Statistics are extracted from LMMs with cell count and mtDNAcn included as fixed factors.

Following this recent publication, I tested if such an association could be found in the dataset of **Chapter II**. Indeed, some mitochondrial metabolic rates were not significantly associated with blood cell counts (Fig.17). However, as shown in the Fig.17 - they were significantly associated with mitochondrial density. When possible, mitochondrial content per cell should thus be assessed to correctly interpret variations in RBC mitochondrial metabolism.

This notion is strongly supported by the finding presented in Chapter I that *in ovo* CORT elevation decreased

most of the mitochondrial metabolic rates by downregulating mitochondrial density. In my thesis, I used mitochondrial DNA copy number as a proxy for mitochondrial density, but this methods is also subject to debate, as it does not address mitochondrial functionality, and is influenced by mitochondrial biogenesis (Medeiros, 2008). Other techniques allow the measurement of mitochondrial content per cell and provide information on mitochondrial functionality, such as measuring the content or expression of the enzyme citrate synthase or cytochrome C (Nord et al., 2021).

(5) A recurring theme through my thesis was the assessment of the impact of a modulation in RBC mitochondrial metabolism on the offspring phenotype during the growth period. To be able to study the complete nestling growth patterns, we monitored individuals that survived until fledging. Therefore, we should keep in mind that our data represent a subsample of nestlings that survived and not the whole population. For **Chapters I and II**, the same applies to the juveniles recaptured a few months after the experiment. Results obtained on juveniles reflects the individuals that first survived, but also individuals that were recaptured, which may introduce a selective bias in our sample. Prospective cohort design would offer a fascinating opportunity to alleviate this bias and further understand how mitochondrial metabolism shapes growth and survival under different environmental conditions.

4.3 Is red blood cell mitochondrial metabolism a good predictor of environmental stress?

Overall, the results of my thesis show that RBC mitochondrial metabolism cannot, by itself, indicate a stressful situation, but should be interpreted in the light of complementary phenotypic traits, such as individual body mass and size. Indeed, in **Chapters II and IV,** individuals that experienced a stressful situation and expressed higher RBC mitochondrial metabolic rates also had a lower body mass and size compared to other individuals. Monitoring the morphometric phenotype provides a good first indication of whether the offspring experienced stressful conditions during the growth period. However, measuring RBC mitochondrial metabolism provides much needed information on the mechanisms underlying phenotypic differences

between individuals. Measuring metabolism and understanding its relationship with animal performance has been a research interest for years, but it is still a field in progress and that interest is not likely to fade soon (Heine & Hood, 2020; Koch et al., 2021).

Because of their contrasting breeding cycles and the different challenges encountered by the offspring during the growth period, I selected two study species to explore the different research questions in my thesis. This choice enabled me to study the variation in RBC mitochondrial metabolism according to environmental conditions within two independent study systems, each with its own challenges. However, a comparative approach by including additional species (e.g. phylogenetically close and distant species) would bring much needed knowledge in the field and extend the potential for generalisation of the results.

Investigating the variation in RBC mitochondrial metabolism in wild species is clearly a relevant approach, as it allows measuring the mechanisms through which the environment influences metabolism *in natura*. However, because the exact environmental determinants of RBC mitochondrial metabolism are currently poorly understood, studying variation in mitochondrial respiration under non-controlled conditions remains challenging. Causal links can be difficult to interpret and conclusions difficult to draw, as the pattern of variation in RBC mitochondrial metabolism may represent a specific situation (a combination of specific environmental conditions) and not a general pattern. Further complementary research conducted in controlled conditions, or, ideally, conducted in parallel in the wild and in control conditions, will therefore continue to be needed to comprehensively interpret the effects found *in natura*.

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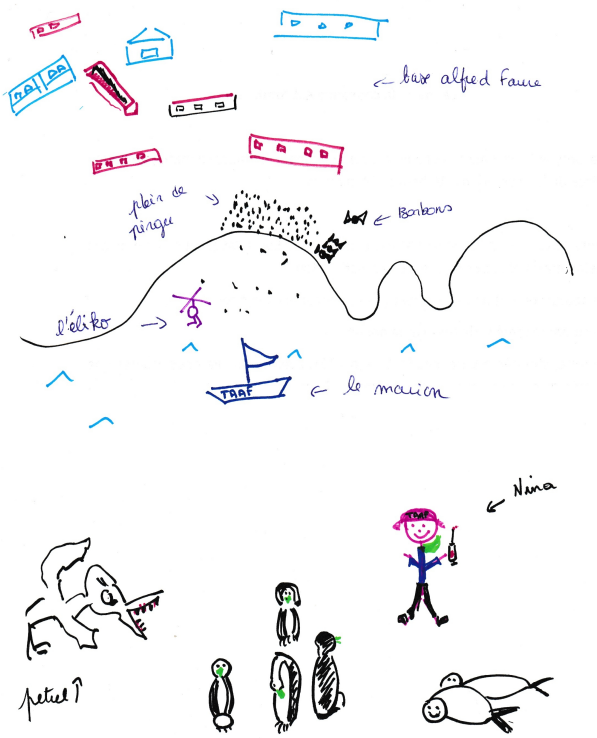
Helsinki, May 2024
Nina Cossin-Sevrin



© This art piece has been paint by an incredible artist: my mom

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