Intergenerational costs of reproduction: Comparative and empirical studies in mammals

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

(Signature) 

Abstract

Life-history theory is built upon the principles of resource allocation and trade-offs. While intraindividual trade-offs have received the most attention, intergenerational trade-offs could be equally essential to our understanding of life-history evolution. One of the most well-studied trade-offs is that of reproduction and survival, known as the 'cost of reproduction'. Currently, oxidative stress is thought to explain this cost but could be paid for by offspring as well as mothers and possibly transferred via several pathways. If oxidative stress can have negative intergenerational implications, then mothers should make some effort to mitigate against this to maximise their fitness. In this thesis, I explore that possibility using a comparative approach examining the evolutionary transitions and life-history associations of one organ that may be responsible for perpetuating intergenerational effects – the eutherian placenta. Repeated evolutionary transitions away from high levels of placentation and associations between high levels of placentation and smaller body masses/shorter gestation lengths provide some evidence for a protective function of the placenta against negative intergenerational effects. To complement this study, I examined the potentially negative intergenerational consequences of the oxidative cost of reproduction. This was done by testing the oxidative cost, constraint and shielding hypotheses in parallel using a supplementary feeding experiment in wild banded mongooses, *Mungos mungo*. While we found that supplementary feeding did not influence oxidative state, we were able to find some support for each of these hypotheses. Of particular interest was the finding that a specific marker for oxidative damage was lower in pregnant females compared to non-breeders, and negatively associated with pup survival to one year. This is suggestive of a deleterious intergenerational effect as a

consequence of oxidative stress and emphasises a need to mitigate against such effects to maximise fitness through mechanisms such as oxidative constraint and shielding. The wide diversity of placentation that we see across mammals may reflect some of the other strategies used by mammals to further minimise negative intergenerational effects and maximise fitness. Ultimately, placentation could play a significant role in the transfer of intergenerational effects and facilitate life-history evolution.

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Author's declaration

Chapters 1 and 4 were solely written by Elsa Evans, with comments and minor edits provided by Jon Blount.

Chapter 2 was designed by Elsa Evans, Jon Blount, Mike Cant and Andy Russell. Elsa Evans wrote the first draft of the manuscript, with comments from Jon Blount and Mike Cant. Data collection was performed by Elsa Evans, and data analysis was conducted by Elsa Evans, Tom Currie and Iain Stott.

Chapter 3 contributions include Magali Meniri, Faye Thompson, Harry Marshall, Rufus Johnstone, Mike Cant and Jon Blount who planned and supervised data collection. Magali Meniri, Lauren Holt, Emma Davey and Christopher Mitchell performed sample analysis. Elsa Evans measured foetus size, wrote methods for the ultrasound protocol, and commented on the whole draft. Hazel Nichols and Gina Lewis constructed the pedigree. Magali Meniri and Jon Blount analysed data and wrote the first draft of the manuscript. All authors gave approval for publication. This paper is submitted for review at the journal Ecology and Evolution at the time of this thesis submission.

Definitions

Antioxidants: Molecules that are ingested or manufactured by the body, which can neutralise free radicals such as ROS.

Brownian motion: From an evolutionary perspective, Brownian motion is used to model the evolution of a continuously valued trait over time. Sometimes called the “random walk” model.

Chorion: A double-layered foetal membrane formed by the trophoblast (see definition), the outermost one, which goes on to form the foetal part of the placenta.

Cotyledonary shape: Eutherian placentas come in 4 different shapes which describe the distribution of placental villi across the chorion. Cotyledonary placentas have evenly spaced groups of villi scattered across the chorion. The other shapes are discoid (seen in humans) where villi occupy a disk-shaped area of the chorion, zonary, where a continuous band of villi encircles the equator of the chorion, and diffuse, where villi occur across the entire chorion.

Endotheliochorial: This is the moderately invasive placenta (see definition for invasion), where the foetal placental tissue erodes through the uterine epithelium and comes into contact with the endothelial lining of the uterine capillaries.

Endothelium: The thin membrane that lines the inside of blood vessels, heart, and lymph vessels.

Epitheliochorial: This is the least invasive placental form (see definition for invasion), where the epithelium of the foetal chorion is in direct contact with the uterine epithelium. No invasion of maternal tissue occurs.

Epithelium: This is a thin protective tissue layer that covers any free surface of the body, or lines a tube or cavity.

Genomic imprinting: Genes from one parent are favourably expressed over those from the other parent, which are epigenetically silenced.

Glutathione: An antioxidant molecule, manufactured by cells in the body.

Hemochorial: The most invasive placental form (see definition for invasion), where the foetal tissue erodes through into the maternal vasculature and the fetal tissue is bathed directly in maternal blood.

Interdigitation: Placental interdigitation describes the spatial arrangement of maternal and foetal tissues, which roughly equates to the surface area available for exchange. There are three main types; labyrinthine, trabecular, and villous (see definitions).

Intergenerational effects: These occur when the effects of an individual's actions carry forwards to the next generation.

Invasion: Placental invasion (invasiveness, interhaemal barrier, or placental interface) represents the maternal-foetal separation type. This is the degree by which maternal and foetal blood are separated from each other. There are three main types; hemochorial, endotheliochorial and epitheliochorial (see definitions).

Labyrinthine: This placental form is the highest degree of placental interdigitation and represents the greatest surface area available for exchange.

Lambda: Otherwise notated as the Greek letter 'λ', this denotes the phylogenetic signal of a model and is a value between 0-1, where 0 indicates no phylogenetic signal and 1 indicated Brownian motion (see definition).

Malondialdehyde: Or MDA, is a marker for the oxidative damage (see definition) of lipid molecules, which is present in plasma.

Matrotrophy: A mode of embryonic development where nourishment is not limited to the yolk, but supplemented by maternally derived nutrients (e.g., through the placenta).

Organogenesis: The formation of organs during embryonic development early in gestation.

Oxidative damage: Damage caused to proteins, lipids, and DNA as a consequence of oxidative stress (see definition).

Oxidative stress: Occurs when the body's antioxidant defence gets overwhelmed by ROS (see abbreviation and definition).

Placentotrophy: A synonym of matrotrophy (see definition).

Pluripotent: Capable of giving rise to several different types of cells. Stem cells are pluripotent.

Protein carbonyls: Or PC, a marker for the oxidative damage of protein molecules.

Reactive Oxygen Species: or ROS, are highly reactive molecules/free radicals derived from oxygen.

Superoxide dismutase: or SOD, is an antioxidant enzyme.

Trabecular: This placental form is the intermediate form of placental interdigitation (see definition) and represents a moderate surface area available for exchange.

Trophoblast: Cells that form the outer layer of the blastocyst (a distinctive stage of the mammalian embryo) that goes on to form the placenta.

Vertical pathogen transmission: Pathogens passed from mother to offspring, either before or after birth via pathways such as the placenta or milk.

Villous: This placental form is the lowest degree of placental interdigitation (see definition) and represents the smallest surface area available for exchange.

Abbreviations

AIC	Akaike Information Criterion
ARD	All rates different evolutionary model
BM	Body mass
DNA	Deoxyribonucleic acid
DNTB	5,5’-dithio-bis-2-nitrobenzoic acid
ER	Equal rates evolutionary model
GL	Gestation length
GSH	Glutathione
H_2O MQ	Milli-Q water
HPLC	High performance liquid chromatography
LS	Litter size
MDA	Malondialdehyde
Mer	Meristic evolutionary model
MI	Matrotrophy index
ML	Maximum longevity
MLik	Maximum likelihood
O_2	Oxygen
ORDERED/ORD	Ordered evolutionary model

OSTA	Oxidative Stress Theory of Aging
PC	Protein carbonyls
PCA	Principal component analysis
PGLS	Phylogenetic generalised least squares
RBC	Red blood cells
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	2- thiobarbituric acid
TEP	1,1,3,3-tetraethoxypropane
TNB	5-thio-2-nitrobenzoic acid
WSMR	Weight-specific metabolic rate
λ	Lambda, quantifies phylogenetic signal

Chapter 1

General Introduction

Life-history theory is built upon the principles of resource allocation and trade-offs (Kavanagh and Kahl, 2016). While intraindividual trade-offs have gained the most attention, Stearns points out that intergenerational trade-offs have been somewhat neglected, despite being potentially as essential to life-history theory as intraindividual trade-offs (Stearns, 1989). Intergenerational effects occur when the effects of an individual's actions carry forward to the next generation, thus not only influencing its own reproduction and survival, but that of its offspring, and potentially many future generations (Andersson, 1978; Livnat, Pacala and Levin, 2005; Plaistow, Lapsley and Benton, 2006). In recent years, this concept has slowly gained more traction, securing interest amongst evolutionary biologists. However, intergenerational effects have largely been considered as adaptive mechanisms, rather than potentially deleterious (Shea, Pen and Uller, 2011; Uller and Pen, 2011; Leimar and McNamara, 2015; Vitikainen *et al.*, 2016). Intergenerational effects may also influence the evolution of species' life-histories. In this thesis, I explore the potentially negative consequences of intergenerational effects by examining the trade-off between reproduction and survival, known as the 'cost of reproduction'. I also investigate patterns of evolution of one organ that may be responsible for the transmission of such intergenerational effects – at least in eutherian mammals - the placenta.

Life-history theory and the cost of reproduction

It has been extensively documented that trade-offs occur between reproduction and lifespan, both across taxa (Kirkwood & Rose, 1991) and within species (Hammers *et al.*, 2013). An individual's chances of survival to a future reproductive attempt and future reproductive success are decreased by a higher level of investment in the present breeding attempt (the so-called 'cost of

reproduction'; Williams, 1966; Stearns, 1992). In an optimal world, species would gain the highest fitness if they both lived a very long time and produced many offspring; however, this is rarely the case. Instead, we observe a trend whereby species fall somewhere along the spectrum of two opposing strategies; species that produce many offspring and have a short lifespan (r-selected species, or fast pace of life), or species that produce few offspring and have a long lifespan (K-selected species, or slow pace of life); this has been dubbed the fast-slow continuum (MacArthur, 1962; MacArthur and Wilson, 1967; Pianka, 1970; Jeschke and Kokko, 2009). Consequently, reproduction is considered a costly endeavour for organisms. Yet there is still considerable debate as to the identity of the proximate mechanism/s responsible for the cost of reproduction.

Oxidative stress in association with breeding is a leading candidate mechanism to explain the trade-off between reproduction and survival. Reactive oxygen species (ROS) are unstable molecules generated as a by-product of normal metabolism that can damage other biomolecules such as DNA, lipids, and proteins (Costantini, 2008; Dowling and Simmons, 2009; Monaghan, Metcalfe and Torres, 2009; Blount *et al.*, 2016). When the body's antioxidant defences are overwhelmed by ROS, this is known as the state of oxidative stress. Energetically costly activities such as reproduction could cause an increase in oxidative damage and loss of cell function that decreases survival over time (Harman, 1956; Beckman and Ames, 1998; Selman *et al.*, 2012). Consequently, oxidative damage could be an important mechanism that underpins senescence, a phenomenon dubbed 'the oxidative stress theory of ageing' or OSTA (Harman, 1956; Sohal and Weindruch, 1996; Beckman and Ames, 1998; Bokov, Chaudhuri and Richardson, 2004; Halliwell and Gutteridge, 2007; Selman *et al.*, 2012). This

could provide a mechanism to explain Kirkwood's disposable soma theory (Kirkwood, 1977; Kirkwood and Holliday, 1979; Dowling and Simmons, 2009; Selman *et al.*, 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). Indeed, several studies have reported positive associations between reproductive effort and oxidative stress, suggesting an oxidative cost to reproduction (Wiersma *et al.*, 2004; Bertrand *et al.*, 2006; Nussey *et al.*, 2009; Bergeron *et al.*, 2011; Garratt *et al.*, 2011; Plumel *et al.*, 2014; Xu *et al.*, 2014; Dupoué *et al.*, 2020). Hereafter, the idea that oxidative stress due to reproductive effort represents a cost to a breeder will be referred to as the 'oxidative cost' hypothesis.

However, evidence that breeding increases an individual's level of oxidative damage remains equivocal (reviewed by Speakman and Selman, 2011; Selman *et al.*, 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). This confusion in the literature has led to the development of new, alternative hypotheses to explain the observed trends. These further hypotheses are not necessarily mutually exclusive from the oxidative cost hypothesis (Stier *et al.*, 2012), and stem from the same mechanistic principles. Some studies found that individuals who exhibited higher levels of oxidative stress prior to breeding saw a lower reproductive output as a consequence (Costantini, Carello and Fanfani, 2010; Stier *et al.*, 2012; Costantini *et al.*, 2016; Montoya *et al.*, 2016), leading to the development of the 'oxidative constraint' hypothesis (Dowling and Simmons, 2009; Stier *et al.*, 2012). However, not all studies find this to be the case (Costantini *et al.*, 2015). In addition to this, other research attempting to find a cost of reproduction found that breeders exhibited higher levels of oxidative stress compared to non-breeders (Stier *et al.*, 2017), whilst several studies find the

opposite trend (Bokov, Chaudhuri and Richardson, 2004; Garratt *et al.*, 2011; Ołdakowski *et al.*, 2012; Garratt, Pichaud, *et al.*, 2013; Costantini, 2014; Schmidt, Blount and Bennett, 2014; Viblanc *et al.*, 2018). This assortment of seemingly contradictory results inspired some to question the experimental design of these studies, reconsider the simplistic trade-off model, and interpret previous results with caution (Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014).

In response to this, Blount *et al*. conducted a meta-analysis in 2016 to search for relationships between reproduction and oxidative damage. The meta-analysis revealed that while oxidative damage is positively correlated with reproductive effort across females of various species of birds and mammals (Garratt *et al.*, 2011; Stier *et al.*, 2012; Yang *et al.*, 2013; Speakman and Garratt, 2014), there is a stepwise reduction in oxidative damage in breeding compared to non-breeding females (Garratt *et al.*, 2011, 2012; Ołdakowski *et al.*, 2012; Garratt, Pichaud, *et al.*, 2013; Yang *et al.*, 2013; Xu *et al.*, 2014). Consequently, the 'oxidative shielding' hypothesis was developed to explain this apparent paradox. Blount et al. suggested that 'oxidative shielding', i.e. a pre-emptive decline in damage levels in breeders, may serve to protect physiologically-dependent offspring from harm caused by increasing levels of oxidative insults over the course of a breeding event (Blount *et al.*, 2016). Supporting evidence for this hypothesis was published in 2016 in a paper by Vitikainen *et al.*, who found that banded mongoose mothers exhibited decreased plasma levels of malondialdehyde (MDA, a marker for oxidative lipid damage) during pregnancy. Moreover, females with higher plasma levels of MDA carried fewer foetuses and had reduced litter survival to emergence from the den (Vitikainen *et al.*, 2016). These results, along with several others, support the idea that maternally derived

oxidative stress during breeding has a negative impact on reproductive fitness which can have intergenerational implications (Bize *et al.*, 2008; Møller, Karadas and Mousseau, 2008; Essa *et al.*, 2015; Dupoué *et al.*, 2020). Potentially, costs of reproduction are not paid wholly by parents, but by their offspring too. However, more data are needed to evaluate the generality of this phenomenon. Much attention has been given to oxidative damage at an individual level (Bokov, Chaudhuri and Richardson, 2004), but until recently, data on intergenerational consequences of oxidative stress were lacking. It is imperative to build intergenerational effects into life-history theory in order to gain a full picture of the consequences of oxidative damage and how this may shape life-history evolution.

Mammalian Placentation

The mammalian placenta is primarily considered an organ of nutrient/oxygen extraction and waste removal functioning for the benefit of the developing foetus (Mossman, 1987; Burton and Fowden, 2015). It is a diverse, transient organ composed of foetal genetic material that embeds into the uterine lining, allowing the exchange of molecules between maternal and foetal blood (Haig, 1993). Mossman states that “The placenta is probably more variable in structure than any other mammalian organ”(Mossman, 1987), whereby eutherian mammals exhibit very different gross placental morphology. If the placental function is supposedly uniform, the evolution of such morphological variation demands an explanation.

The driving force behind placental evolution remains ambiguous; for many years, the intimate relationship between a mother and her dependent foetus was considered a harmonious one; both mother and foetus have the same interests due to the shared genes they carry (Haig, 1993). This is now considered as the 'cooperative/altruistic hypothesis' (Banet, Au and Reznick, 2010; Moore, 2012). Trivers was the first to recognise that this relationship might not be so harmonious. A mother and her offspring only share half of their genes leaving room for disagreement over parental investment (Trivers, 1974). This idea was further developed by Haig who provided examples of how this may occur in human pregnancy, and thus the 'parent-offspring conflict hypothesis' was born (Trivers, 1974; Haig, 1993). It is thought that parent-offspring conflict can influence placentation via genomic imprinting (Haig, 1992). This occurs when genes from one parent are favourably expressed over the other, which are epigenetically silenced. Regarding placentation, it is thought that the paternal genes which control foetal growth are favourably expressed over the maternal genes, theoretically, this demands an increase in parental investment from the mother to her detriment (Haig, 1992). It is thought that this conflict of interests may drive the divergent evolution of placentation, and explain the diversity of placental traits we see in nature (Haig, 1993).

The mammalian placenta can be classified in a variety of different ways. The placental shape is perhaps the most striking difference, but other differences include vascular arrangement, interhaemal barrier (degree of invasiveness, or placental interface), and maternal-foetal interdigitation (Knobil and Neill, 2006) (Leiser and Kaufmann, 1994). Regarding placental morphology and its impact on developing foetuses, placental invasiveness has been the most widely studied

(Capellini, Venditti and Barton, 2011). The gross placental morphology of most eutherian placentas falls into three main categories of invasiveness as defined by Grosser: hemochorial, endotheliochorial and epitheliochorial (Grosser, 1909). Epitheliochorial placentas are considered the least invasive type, whereby the epithelium of the fetal chorion rests against the uterine epithelium and all the maternal tissue layers are retained. Endotheliochorial placentas are considered moderately invasive, where the epithelium of the chorion erodes through the uterine epithelium and is in contact with the endothelial cells of the maternal blood vessels. The most invasive form of the placenta is the hemochorial placenta, where the foetal chorion invades the maternal vasculature and is bathed directly in maternal blood (Mossman, 1987; Leiser and Kaufmann, 1994; Wooding and Burton, 2008; Capellini, Venditti and Barton, 2011; Vaughan, Ryan and Czaplewski, 2011). Previous research suggests that more invasive forms of the placenta could aid resource acquisition by the foetus via mechanisms such as hormonal manipulation (Haig, 1993; Petry, Ong and Dunger, 2007; Elliot and Crespi, 2008), and also increase the rate of substance transfer from maternal to fetal blood (Haig, 1993; Elliot and Crespi, 2008; Vaughan, Ryan and Czaplewski, 2011).

When researching the impact of placental morphology on developing foetuses, placental interdigitation has been largely overlooked. However, Capellini and her team have suggested that it could be equally, if not more important than invasiveness (Capellini, Venditti and Barton, 2011; Capellini, 2019). Interdigitation can be thought of as the type of maternal-foetal connection and roughly equates to the surface area available for exchange (Wooding and Burton, 2008). Three distinct categories of interdigitation are recognised; villous,

trabecular and labyrinthine (Mossman, 1987; Mess and Carter, 2006), although two other intermediate forms have been documented (Leiser and Kaufmann, 1994; Mess and Carter, 2006; Wildman *et al.*, 2006; Capellini, Venditti and Barton, 2011). Labyrinthine interdigitation represents the largest surface area available for exchange and is thus expected to provide the foetus with higher rates for nutrient transfer (Capellini, Venditti and Barton, 2011). Villous interdigitation represents the smallest surface area for exchange, whilst trabecular is intermediate. Therefore, increased placental invasiveness and interdigitation were predicted to provide the foetus with greater access to resources, and therefore should increase offspring growth rate. While they found this to be true, Capellini and her team noted that interdigitation was the key variable that correlates with gestation length (Capellini, Venditti and Barton, 2011).

Research into factors driving placental evolution has mainly focussed on finding associations between placentation and life-history traits. Studies on *Poeciliopsis* species of fish, which display varying degrees of placentation, have found increased placentation to be associated with earlier age at sexual maturity and higher rates of reproduction earlier in life (Pires, McBride and Reznick, 2007; Pires *et al.*, 2011). This is indicative of higher placentation being associated with a faster pace of life, or an r-selected life-history strategy, which led to the development of the 'life-history facilitation' hypothesis (Pires *et al.*, 2011). This hypothesis suggests that evolutionary changes in life-history traits could drive the evolution of placentation or *vice versa* (Pires *et al.*, 2011). Further research into mammalian placentation has also revealed distinct 'eutherian constellations', which associate a fast pace of life with increased placental invasion and

interdigitation (Lewitus and Soligo, 2011). Similarly, Garratt, Gaillard, *et al.* (2013) found a high degree of interdigitation to be associated with a faster pace of life. However, once the extent of interdigitation was statistically controlled for, increased placental invasion was in fact associated with a slower pace of life (Garratt, Gaillard, *et al.*, 2013).

It has been assumed that the hemochorial placenta is the most highly evolved, as it is the placenta type exhibited by humans (Mossman, 1937, 1987; Haig, 1993; Carter, 2001; Vogel, 2005). However, recent phylogenetic analysis has revealed that some form of invasive placentation and labyrinthine interdigitation is likely the ancestral state of mammals (Elliot and Crespi, 2006, 2009; Wildman *et al.*, 2006). While it is wholly agreed that the least invasive epitheliochorial placenta is derived, there remains some debate over whether the ancestral state of placental invasiveness is hemochorial or endotheliochorial (Carter and Enders, 2004; Vogel, 2005; Mess and Carter, 2006, 2007; Carter and Mess, 2007; Martin, 2008). Regardless, the evolution of less invasive and less interdigitated placenta types must have subsequently arisen to serve an adaptive function. This is where the 'cooperative/altruism hypothesis' falls short; it cannot explain the transition away from a higher degree of invasion or interdigitation. The 'parent-offspring conflict' hypothesis provides a rational answer whereby the loss of invasiveness and interdigitation could serve as an advantage to the mother where she may be able to reduce the degree of manipulation from the foetus (Vogel, 2005). However, if greater placental invasiveness and interdigitation are linked to greater resource acquisition from maternal blood, then it is plausible that greater intergenerational transfer of oxidative damage could also occur in more invasive or interdigitated placental types (Blount et al. 2016). This could

potentially explain why the evolution of less invasive, less interdigitated placentas arose.

Oxidative stress in mothers may produce intergenerational consequences through a variety of mechanisms beyond the direct transmission of ROS across the placental barrier. ROS are known to damage lipids (Costantini, 2008; Dowling and Simmons, 2009; Monaghan, Metcalfe and Torres, 2009; Blount *et al.*, 2016), which are a secondary energy source for developing foetuses after carbohydrates (Père, 2003). Indeed, lipids in the form of fatty acids have been observed to readily transfer from maternal blood across the placental barrier to the foetus (Koren and Shafrir, 1964; Hershfield and Nemeth, 1968; Edson, Hudson and Hull, 1975; Hummel, Schirrmeister and Wagner, 1975; Elphick, Hudson and Hull, 1975; Elphick and D Hull, 1977; Elphick and D. Hull, 1977; Hull and Elphick, 1979; Thomas and Lowy, 1982, 1983, 1984; Gilbert, Hauguel and Bouisset, 1984; Hendrickse, Stammers and Hull, 1985; Stephenson, Stammers and Hull, 1990; Honda, Lowy and Thomas, 1990; Haggarty *et al.*, 1997; Portman, Behrman and Soltys, 2017), while fatty acid transfer in species with non-invasive placentation is minimal to non-existent and most fatty acids have to be manufactured by the liver of the foetus (Elphick, Hull and Broughton Pipkin, 1979; Shand and Noble, 1979; Elphick *et al.*, 1980; Leat and Harrison, 1980; Elphick and Hull, 1984; Thulin *et al.*, 1989; Père, 2001). Then, hemochorial placentation represents a possible pathway for the transmission of oxidised lipids, not only would this potentially lower the nutritional quality of the maternal resources or the overall transfer of nutrients but may also cause lipid peroxidation in the foetus to its detriment (Serini *et al.*, 2011). Oxidative stress in humans has been linked to a variety of pregnancy-related disorders including preeclampsia, foetal growth

restriction, spontaneous abortion, embryopathies, altered placental vasculature, low birth weight and preterm labour (Gupta *et al.*, 2007; Al-Gubory, Fowler and Garrel, 2010). It has previously been hypothesised that human placentation limits the exposure of foetuses to oxygen (O_2) during early development when the embryo is most sensitive to oxidative damage (Burton, Hempstock and Jauniaux, 2003; Jauniaux, Gulbis and Burton, 2003). While lethal effects of oxidative stress will have fitness implications for the parents, sub-lethal effects of oxidative stress are more likely to be relevant to the perpetuation of negative intergenerational effects.

Thesis outline

Logically, placentation should be intrinsically connected to the cost of reproduction. From an intragenerational perspective, placentation may govern the degree of maternal exploitation, which is likely to be incurred as an oxidative cost to the mother or excess use of resources that trades off with her survival. From an intergenerational perspective, this greater access to resources may come with the inevitable consequence of greater damage transmission. If maternally derived oxidative damage has intergenerational implications to the extent where it could influence a species' life-history (Bokov, Chaudhuri and Richardson, 2004), it would be plausible for a mother to invest in a system to buffer her foetuses against deleterious oxidative assaults. This may increase her offspring's probability of survival and therefore enhance the mother's evolutionary fitness. The evolutionary step towards less invasive, less interdigitated placentas in some mammalian lineages could potentially be an adaptive feature exhibited by species that are more at risk of intergenerational damage.

The association between mammalian life-histories and placentation has been observed multiple times as previously discussed (Pires, McBride and Reznick, 2007; Lewitus and Soligo, 2011; Pires *et al.*, 2011; Garratt, Gaillard, *et al.*, 2013), particularly where an increased level of placentation/invasiveness is associated with a faster pace of life (Pires, McBride and Reznick, 2007; Lewitus and Soligo, 2011; Pires *et al.*, 2011). As oxidative damage has been proposed to mediate the trade-off between reproduction and survival, this association between placentation and life-histories could be mediated by the same mechanism. Thus, there is potential for the oxidative shielding hypothesis to be the connecting piece of the puzzle, drawing together these two disciplines to gain a better understanding of life-history evolution in Eutherian mammals.

In this thesis, I explore the novel idea that placental morphological variation could have arisen as a way of buffering foetuses against potentially harmful molecules that could also be transferred across the placenta from maternal blood (Chapter 2). It has previously been suggested that placentation could play a protective role (Loke, 1982, Webster and Kapel, 2005 and Capellini, Nunn and Barton, 2015), whereby lower degrees of invasion could have evolved to limit vertical pathogen transmission between maternal and foetal blood. However, there is limited evidence to support this idea (Loke, 1982; Webster and Kapel, 2005; Capellini, Nunn and Barton, 2015). Vertical transmission of oxidative damage could also provide an explanation for a protective function for the placenta. I explore whether placental form may have evolved in relation to risk of intergenerational harm using the largest phylogenetic comparative analysis to date of 732 species of eutherian mammal, examining associations between placental traits and various life-history characteristics. In conjunction with that, trait switching analyses and ancestral

state reconstructions on the same dataset are used to infer the order of evolution and the ancestral state of the mammalian placenta. These placental analyses will test the 'protection' hypothesis alongside the 'cooperation/altruism' hypothesis and 'parent-offspring conflict' hypotheses, to elucidate whether there is any evidence for a protective role as a consequence of oxidative stress.

To complement that, I further investigate the cost of reproduction by testing the oxidative cost, constraint and shielding hypotheses in parallel (Chapter 3). This study will experimentally manipulate oxidative damage/defence via supplementary feeding of female Ugandan banded mongooses, *Mungos mungo*, whose life-histories and those of their offspring have been documented over 35 months, alongside markers for oxidative damage and defence to determine whether we find further evidence for intergenerational damage and oxidative shielding. The banded mongoose population on the Mweya Peninsula in Queen Elizabeth National Park, Uganda, is an excellent system to test these hypotheses. This wild population has been studied since 1995 and detailed records of each individual's life-history have been continuously collected (Cant, Vitikainen and Nichols, 2013; Cant *et al.*, 2016). Although wild, much of this population has been habituated to the presence of humans, making the monitoring of such fine-scale life-history data possible. This wild population also allows for the study of real trade-offs between reproduction and survival that are not present in a captive population. Banded mongooses live in mixed-sex social groups of between 5-25 adults, and breed on average four times per year, where litters are born synchronously and all adults raise the offspring communally (Cant, 2000; Cant, Vitikainen and Nichols, 2013; Cant *et al.*, 2016). This system can facilitate a powerful split-plot experimental design, where maternal nutrition can be

manipulated via dietary provisioning in the treatment females. Maternal investment and offspring survival can be compared to control females, where all individuals are exposed to the same environmental conditions throughout the experiment. The banded mongooses have also been previously used to test the oxidative cost of reproduction; in fact, it was this exact study system that first identified empirical evidence for oxidative shielding (Vitikainen *et al.*, 2016), making it an ideal candidate for this experiment on the cost of reproduction and its influence on offspring survival.

Chapter 2

Why is placental form so variable? A phylogenetic comparative analysis of alternate hypotheses

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Abstract

The diversity of the mammalian placenta is surprising given its primary function of nutrient and waste transfer. The most popular explanation for this diversity is that parent-offspring conflict has driven increases and decreases in placental invasiveness and interdigitation (surface area). Alternatively, variation in placental form may have been shaped by evolutionary pressure to mitigate against intergenerational harm caused by the maternal-offspring transfer of pathogens or oxidative stress. Here we investigate these hypotheses using comparative phylogenetic analysis of 732 mammals for which information on life-history and placental form was available. Our results show that both increased placental invasiveness and interdigitation are associated with shorter gestation lengths. Furthermore, larger body mass was found to be associated with lower degrees of both placental invasion and interdigitation. Trait switching models and ancestral state reconstructions indicated that the ancestral state of the placenta was endotheliochorial (moderate invasion) and labyrinthine (highly interdigitated). We suggest that transitions away from higher invasion and interdigitation provides convincing evidence for a protective function of the placenta. Moreover, associations between placentation, gestation length and body mass point to metabolic facilitation as a possible explanation for diversity in placental form, as a consequence of the allometric scaling of weight-specific metabolic rate. Consequently, the placental form may influence the evolution of life-history traits by the intergenerational transmission of pathogens or oxidative stress.

Key words: placenta, invasion, interdigitation, protection, parent-offspring conflict, life-history, oxidative stress

Introduction

The mammalian placenta is histologically complex and varies widely across species. The best-studied aspect of this variation is placental invasiveness, which describes the distance between maternal and foetal blood (Leiser and Kaufmann, 1994; Knobil and Neill, 2006; Capellini, Venditti and Barton, 2011). Three main categories of placental invasiveness are recognised; the most invasive being hemochorial placentas, and the least invasive being epitheliochorial placentas, while endotheliochorial placentas have intermediate invasiveness (Grosser, 1909; Mossman, 1987; Leiser and Kaufmann, 1994; Wooding and Burton, 2008; Capellini, Venditti and Barton, 2011; Vaughan, Ryan and Czaplewski, 2011). Not only is this thought to influence the rate of nutrient transfer across the placental barrier, but to an extent, may also determine which substances are able to cross the placental barrier (Loke, 1982; Crespi and Semeniuk, 2004; Webster and Kapel, 2005; Elliot and Crespi, 2008; Capellini, Venditti and Barton, 2011). Another important aspect of placental morphological variation is interdigitation, this is the spatial arrangement of maternal and fetal tissue and equates to the surface area available for maternal-foetal exchange (Wooding and Burton, 2008). This is also thought to influence the rate of nutrient transfer and influence foetal growth rates (Wildman *et al.*, 2006; Capellini, Venditti and Barton, 2011; Capellini, 2012). Labyrinthine placentas provide the greatest surface area, while villous placentas have the smallest surface area, and trabecular placentas are intermediate (Mossman, 1987; Mess and Carter, 2006).

The placenta has historically been thought of as a cooperative organ, which has evolved simply to facilitate the transfer of nutrients from mother to offspring, and

the removal of waste products in reverse (placental cooperative provisioning/altruism hypothesis; (Haig, 1993; Moore, 2012)). However, the diversity of the mammalian placenta is puzzling given the apparent simplicity of its role (Mossman, 1987; Burton and Fowden, 2015). It has been assumed that the least invasive placenta type must be the most primitive (Mossman, 1937, 1987; Haig, 1993; Carter, 2001; Vogel, 2005). David Haig proposed the idea that increased placental invasion may have evolved more recently, in order to provide foetuses with greater control over access to maternal resources (Haig, 1993; Crespi and Semeniuk, 2004). This makes intuitive sense because the placenta is defined as an apposition of foetal and maternal tissue, but placental invasion refers to the foetal trophoblast invading through the uterine epithelium (Mossman, 1937; Haig, 1993; Benirschke, 2006; Wooding and Burton, 2008; Reece *et al.*, 2011). Indeed, reference to placental 'invasiveness' implies that there is a conflict between mother and offspring. The influence of parent-offspring conflict on placental evolution could be amplified by genomic imprinting (Haig, 1992). Genomic imprinting is when the inherited genes from one parent are favourably expressed over the inherited genes of the other parent, whose copy of the gene is epigenetically silenced (Haig, 1993; Reik and Walter, 2001). This is significant for mammals, as it is thought to influence nutrient transfer from mother to offspring. The genes that promote foetal growth *in utero* could be one example of paternal genes that are favourably expressed, thus demanding increased parental investment from the mother (Haig, 1992). In monogamous species, fathers will not benefit from increased maternal investment because it will come at the expense of their future offspring. In contrast, the opposite is true for fathers of promiscuous species, as future offspring from that female are likely to be unrelated (Trivers, 1974; Long, 2005). Interestingly, phylogenetic analysis now

suggests that the ancestral state of the placenta was likely hemochorial, or possibly endotheliochorial, and with a labyrinthine form of interdigitation (Vogel, 2005; Mess and Carter, 2006, 2007; Wildman *et al.*, 2006; Carter and Mess, 2007; Martin, 2008; Elliot and Crespi, 2009). Perhaps then, the emergence of less invasive placentas and lower degrees of interdigitation over more recent evolutionary history has allowed mothers to wrest back control of their resources (Capellini, Venditti and Barton, 2011; Garratt, Gaillard, *et al.*, 2013).

An alternative idea is that the placenta has the potential to protect offspring against any detriment which may come from the mother. Specifically, the placental form may reflect the need to mitigate against the risk of deleterious intergenerational effects ('placental protection' hypothesis). Such harm could manifest in various ways. For example, vertical transmission of pathogens from mother to foetus is a well-documented phenomenon and is known to compromise both maternal and foetal fitness (Loke, 1982; Goldenberg, Hauth and Andrews, 2000; Robbins and Bakardjiev, 2012). It has previously been suggested that evolution away from high placental invasion could serve to reduce pathogen transmission (Loke, 1982; Webster and Kapel, 2005; Capellini, Nunn and Barton, 2015). There is currently only limited empirical evidence to support this pathogen protection hypothesis (Capellini, Nunn and Barton, 2015). Furthermore, it remains unclear how rates of substance transfer across the placental barrier determined by placental interdigitation, would protect foetuses in utero from vertical pathogen transmission.

Another possible avenue for placental protection is that placental form has evolved to reduce intergenerational harm caused by oxidative stress. Investment in breeding incurs greatly increased metabolic rate, and concomitant risk of oxidative stress (Kirkwood and Holliday, 1979; Beckman and Ames, 1998; Speakman and Garratt, 2014). Meta-analysis across species has suggested that while the risk of oxidative stress does indeed scale with the level of reproductive effort (i.e., offspring number or size), there is a marked reduction in oxidative damage levels in breeding compared with non-breeding females (Blount et al. 2016). It was suggested that breeders decrease levels of oxidative damage on the transition to reproduction in order to protect themselves and in particular their physiologically-dependent offspring, from harm caused by the inevitable increase in oxidative stress that manifests over the course of a reproductive episode (the 'oxidative shielding' hypothesis; Blount *et al.*, 2016). Indeed, there is extensive evidence from studies of humans that oxidative stress during pregnancy is associated with conditions such as pre-eclampsia, miscarriage and intrauterine growth restriction (Jauniaux, Poston and Burton, 2006; Hoffmann *et al.*, 2008; Burton and Jauniaux, 2011). In banded mongooses, females reduced damage levels during pregnancy, while higher levels of oxidative stress in mothers was found to be associated with smaller litter size and lower pup survival (Vitikainen *et al.*, 2016). While reduced placental invasion may offer a physical barrier to oxidative damage transmission, placental interdigitation may offer an important protective function from oxidative damage too. In theory, a slower rate of substance transfer across the placenta means a slower exposure of the foetus to oxidative damage. Wildman et al. suggest that villous interdigitation could entail a lower metabolic demand on the mother than labyrinthine interdigitation, allowing her to sustain longer gestation periods (Wildman *et al.*, 2006). This could

potentially be a symptom of parent-offspring conflict allowing mothers greater control over their resources. However, if lower levels of placental interdigitation indeed work to reduce the metabolic demand on the mother, this would theoretically reduce the levels of oxidative stress that she experiences too. Therefore, with lower interdigitation, the mother may be in a better position to maintain oxidative homeostasis and consequently experience less oxidative damage overall, the foetus will then be exposed to this at slower a rate. This could allow the foetus a better capacity to defend itself. Perhaps then, the evolution of less invasive, less interdigitated placentas may be an adaptation to protect foetuses, particularly in species where intergenerational damage has significant potential to reduce fitness (Blount *et al.*, 2016).

Previous studies have found that body mass correlates negatively with the level of placental invasiveness, whereby hemochorial invasion is associated with small body size and epitheliochorial placentation is associated with relatively large body size (Elliot and Crespi, 2008, 2009; Martin, 2008). While this trend has been noted several times, it has been little discussed. Body mass has largely been considered as a correlate of life-history strategies that have coevolved with placental form (Pires, McBride and Reznick, 2007; Lewitus and Soligo, 2011; Pires *et al.*, 2011; Garratt, Gaillard, *et al.*, 2013). Elliot and Crespi (2008) identified that steeper brain-body allometry is associated with more invasive placentation, and they suggested that greater placental invasion facilitates faster prenatal brain growth. Both Martin (2008) and Elliot and Crespi (2009) discuss the use of body mass to infer the ancestral state of the eutherian placenta (Martin, 2008; Elliot and Crespi, 2009). However, none of these papers provide any explanation for

the striking relationship between placental invasion and body mass, nor has an association between placental interdigitation and body mass been tested before.

The potential role of metabolic scaling as a driver of placental form has not previously been considered. Metabolic rate scales allometrically with body mass across species; when considering weight-specific metabolic rate (WSMR), it is clear that small animals have tissues that are far more metabolically active than large animals. The allometric relationship between body mass and metabolic rate is notoriously difficult to explain, but this metabolism-weight relationship encompasses almost every aspect of an animal's physiology (Western, 1979; Schmidt-Nielsen, 1997; Hill, Wyse and Anderson, 2012). A higher degree of placental invasion or interdigitation may facilitate the elevated metabolic demands of developing foetuses in smaller mammals. However, increased placental invasiveness or interdigitation could also mean an elevated exposure to oxidative stress *in utero*. If so, this may only be viable in species that are selected for a faster pace of life. Evolutionary theory envisages a spectrum of life-history strategies, ranging from the production of few 'expensive' offspring (i.e. slow to develop and mature; long-lived) in stable environments, to the production of many 'cheap' offspring (fast to develop and mature; short-lived) in unstable environments (i.e. an r/K selection or fast-slow continuum) (MacArthur, 1962; MacArthur and Wilson, 1967; Pianka, 1970; Jeschke and Kokko, 2009). Body mass is thought to be the causal influence on life-history traits, and thus explain this fast-slow continuum (Western, 1979; Peters, 1983; Schmidt-Nielsen, 1983; Dobson and Oli, 2007). If body mass can be associated with both placentation and life-histories, the question then arises, which evolved first? Placental

invasiveness which imposed selection pressure on the pace of life, or pace of life which imposed selection pressure on placentation?

The association between placentation and life-history strategies has been discussed several times previously (Pires, McBride and Reznick, 2007; Lewitus and Soligo, 2011; Pires *et al.*, 2011; Garratt, Pichaud, *et al.*, 2013). Studies on life-history evolution across *Poeciliopsis* species of fish, which display varying degrees of placentation, suggest that increased placentation (measured by a higher matrotrophy index) may facilitate the evolution of earlier age at maturity and higher rates of reproduction early in life (Pires, McBride and Reznick, 2007; Pires *et al.*, 2011). Similarly, an association analysis between placental structure and life-history traits in eutherian mammals identified two distinct eutherian constellations which represents a divergence of placental physiology and life-history strategies. Similar to the fish research, hemochorial labyrinthine placentation (high degree of invasion/interdigitation) was associated with a fast pace of life, while epitheliochorial villous/trabecular placentation (lower degree of invasion/interdigitation) was associated with a slow pace of life (Lewitus and Soligo, 2011). The same association between interdigitation and life-history was observed by Garratt, Gaillard, *et al*., (2013), but once this was statistically controlled for, epitheliochorial invasion was associated with a fast pace of life (Garratt, Gaillard, *et al.*, 2013).

In the present paper, we readdress the question of which life-history traits may drive placental evolution - or *vice versa*. We used the largest database of mammalian placentation constructed to date, consisting of 732 eutherian

species. We assembled a database of placental invasiveness, interdigitation, maximum longevity, gestation length, litter size, and body mass with representation of all eutherian orders. Using a phylogenetic comparative approach and ancestral state reconstructions we aimed to thoroughly explore the relationships between placental types and life-history traits seated in the context of placental evolutionary transitions. Associations between placental form and life-history were examined in the context of three different hypotheses: the 'cooperation/altruism' hypothesis, the 'parent-offspring conflict' hypothesis, and the 'protection' hypothesis.

These hypotheses are unlikely to be mutually exclusive. However, we can make some predictions about relationships between placental form, life-history traits and direction of evolution, which may help to separate these hypotheses. If placental diversity is the product of cooperation between mother and offspring, we might expect less invasive, less interdigitated placentas to be associated with r-selected species (whose life-history strategies are almost by definition characterised by relatively low parental investment). We might also expect the ancestral state of the placenta to be minimally invasive/interdigitated and only evolve towards a greater degree of invasion/interdigitation. Alternatively, if the placental form has diversified in order to protect offspring from vertical transmission of pathogens or oxidative stress, then we might expect that less invasive and/or interdigitated placentas will be associated with K-selected life-history strategies, as protection should increase with parental investment. The 'protection' hypothesis also predicts that the ancestral state was highly invasive/interdigitated and only transition towards a lower degree of invasion/interdigitation. The parent-offspring conflict hypothesis does not make

clear predictions about associations between life-history strategy and placenta type, nor would we expect a particular ancestral state based upon this hypothesis. However, we would expect a higher degree of placental invasion/interdigitation to indicate foetal control of maternal resources and a lower degree of invasion/interdigitation to indicate maternal control of resources. Therefore, this coevolutionary arms race may lead to more frequent switching back and forth between placenta types, or potentially an initial transition towards greater invasion/interdigitation where the foetus gains an advantage before transitioning back towards lower levels of invasion/interdigitation as the mother regains control over her resources. Finally, based on the concept of genomic imprinting, we predict that monogamous mating systems will be associated with a lower degree of placental invasion/interdigitation, whereas promiscuous mating systems will be associated with a higher degree of invasion/interdigitation.

Methods

Data Collection

A database was compiled consisting of eutherian mammal species and including placental classification and life-history parameters. In total, the database includes 732 species from 21 orders (Afrosoricida, Artiodactyla, Carnivora, Cetacea, Chiroptera, Cingulata, Dermoptera, Erinaceomorpha, Hyracoidea, Lagomorpha, Macroscelidea, Perissodactyla, Pholidota, Pilosa, Primates, Proboscidea, Rodentia, Scandentia, Sirenia, Soricomorpha, Tubulidentata).

Initially, data for placenta type were collated following a literature search for a single species representative for each of the 21 orders of eutherian mammal, according to Wilson and Reeder's 'Mammal Species of the World' (Wilson and Reeder, 2005). This was to ensure that the database was as broad and inclusive as possible. Additional data were then sourced from published comparative analyses (Elliot & Crespi 2008, 2009). Data were available for 367 hemochorial species, 183 endotheliochorial species, and 182 epitheliochorial species. Categories of placental invasiveness were ranked: epitheliochorial<endotheliochorial<hemochorial. Following this, data for placental interdigitation was sourced from the literature (Elliot and Crespi, 2009; Capellini, Venditti and Barton, 2011). These data were ranked in terms of relative surface area: villous<trabecular<labyrinthine. Data for placental categories were cross-referenced with their original sources to determine whether the assigned placenta types are based on species-specific reports or whether they are genus-specific or assumed based on a closely related species.

Life-history data were obtained from the PanTHERIA database (Jones *et al.*, 2009). The recorded life-history parameters are maximum longevity (months), gestation length (days) and litter size (number of offspring per litter per female). Data for all life-history traits were transformed to $\log_{10}$. Further details on how the PanTHERIA team collated and processed their data, along with the database itself, are published electronically in Ecological Archives at http://esapubs.org/archive under the accession number E090_184. Mating systems data was collated based on an exhaustive literature search. Literature was found using Google Scholar and Web of Science with no year limit on the search. Combinations of terms searched included 'mating system' or types of mating system (e.g. 'monogamy' or 'monogamous') coupled either with the term 'mammal(s)', specific mammalian orders or specific species names. For simplicity, and to resolve some discrepancy between sources, mating systems were classified as either monogamous or non-monogamous.

Statistical analyses

Statistical analyses were carried out using the software 'R' version 3.6.1 (R Core Team, 2016) and 'RStudio' (RStudio Team, 2016). P-values less than 0.05 were considered statistically significant. The 'ape' package (Paradis and Schliep, 2019), the 'phylolm' package (Ho and Ane, 2014), the 'phytools' package (Revell, 2012), the 'geiger' package (Pennell *et al.*, 2014), the 'ggplot2' package (Wickham, 2016) and the 'caper' package (Orme *et al.*, 2018) were installed into R Studio in order to conduct this analysis.

Variation in life-history parameters and placental type are likely explained to some extent by the degree of relatedness between species, which could confound any associations identified. Therefore, we tested whether any observed relationships between placental diversity and life-histories remained once phylogeny was controlled for. An almost complete mammal phylogeny was sourced from Bininda-Emonds *et al.* (2007). Ten mammal species for which we had placenta and life-history information were missing from this mammal phylogeny (*Chrysochloris granti*, *Chlorocebus pygerythrus*, *Papio cynocephalus*, *Papio papio, Papio ursinus*, *Myoprocta pratti*, *Sciurus aberti, Felis catus, Geomys bursarius* and *Geomys attwateri*). Subsequently, these 10 species were removed from the phylogenetic analysis.

To thoroughly explore our hypotheses and tease apart any relationships that we identified, we used the statistical models described below:

1) Do placental traits vary with life-history traits?

We compared estimates between basic linear models and phylogenetic generalised least squares (PGLS) models both with and without body mass as an explanatory variable. For these models, λ (which quantifies phylogenetic signal) was calculated using maximum likelihood (MLik) and is given a value between 0-1, where 0 indicates no phylogenetic signal and 1 indicates Brownian motion (Blomberg and Garland, 2002; Freckleton, Harvey and Pagel, 2002). This allowed us to determine whether any trends observed between placenta type and life-history traits are best explained by body mass or phylogeny, and the strength of that phylogenetic signal. Placental invasion or interdigitation was used as an

explanatory variable in separate models, with either maximum longevity, gestation length or litter size as the response variable. For the models in which it was used, body mass was included as a covariate. This technique has been used in previous phylogenetic comparative analyses of placental traits and life-histories (Capellini, Venditti and Barton, 2011; Garratt, Gaillard, *et al.*, 2013). The raw distributions of the life-history traits by placenta type were compared to residual plots from PGLS models with body mass as an explanatory variable that controls for phylogeny. This process was repeated for both placental invasiveness and interdigitation.

2) What is the influence of body mass on the relationship between placental traits and life-history traits?

Associations between body mass and placenta type were tested in a similar way to the other life-history traits, this time using body mass as the response variable and either placental invasion or interdigitation as the explanatory variable. We compared estimates of both basic linear models and PGLS models, again calculating λ using MLik. Models were run either with no covariate, or one of the other three life-history traits as a covariate (maximum longevity, gestation length or litter size). We also included a model for both placental invasion and interdigitation with all three life-history traits together to thoroughly explore the relationships between body mass, life-histories and placentation.

3) Do placental traits vary with mating system?

Mating systems were converted to binary for the sake of simplicity, where monogamy =1 and non-monogamy=0. A Chi-squared test was run to determine if the distribution of placenta type by mating system differed from the expected distribution. Subsequently, both a logistic regression and a phylogenetic logistic regression were run, where mating system was the response variable and placental trait was the explanatory variable. These were repeated with and without body mass as a covariate for both placental invasiveness and interdigitation. To determine which explanatory variables best explained mating system, phylogenetic logistic regression models with combinations of placental invasiveness, placental interdigitation and body mass as explanatory variables were compared using the Akaike Information Criterion (AIC). The null model controlled for phylogeny but contained no explanatory variables. The model with the lowest AIC value was determined as the best fit for the data (Burnham and Anderson, 2002; Wagenmakers and Farrell, 2004). Models were accepted as a better fit if they were 2 or more AIC units lower than other models. If an association was found between placenta type and mating system after controlling for body mass and phylogenetic relatedness, or if models including placenta type were found to explain mating system better than the null model, mating system was used as a fixed categorical factor in the analysis of trait co-variation model comparisons.

4) Do placental traits explain life-history variation better than body mass alone?

Due to statistical limitations, it is currently impossible to use placenta type as a response variable in these phylogenetic models. Instead, we compared PGLS models for each life-history trait, where the explanatory variables are combinations of placental invasiveness, placental interdigitation and body mass. The null model included body mass alone as an explanatory variable, as this is known to explain some variation in life-history traits. For each life-history trait (maximum longevity, gestation length and litter size) the sample size was kept the same so as not to bias any models with different amounts of data. Again, models were compared using AIC, where the model with the lowest AIC value was deemed the best fit for the data and models were accepted as a better fit if they were 2 or more AIC units lower than other models. For simplicity in these comparative models, λ was set to 1 which indicated Brownian motion. This was repeated for maximum longevity, gestation length and litter size.

5) What are the evolutionary transitions of placental traits?

Evolutionary transitions in placental states were determined by a trait switching analysis followed by a simulated ancestral state reconstruction. To explore the direction and rates of change between different placental states, a trait switching analysis was run on both placental interface and placental interdigitation. For each placental trait, four separate evolutionary models were run to establish which model was the best fit for the data. Models tested are the 'Equal Rates' model (ER) which assumes trait switching occurs equally between all traits, the

‘All Rates Different’ model (ARD) where the rates of switching between all traits are independent of each other, the ‘Meristic’ model (Mer) where for trait A to evolve to trait C, it has to evolve via trait B and the switching rates are symmetrical, and the ‘Ordered’ model (ORDERED) similar to the Meristic model, but rates are not symmetrical. Models were compared using AIC values, and the model with the lowest AIC value was chosen as the best fit for the data. The evolutionary model that best fits the data for each placental interdigitation and placental invasion was then mapped onto the phylogenetic tree using the multi-simulation method. This visually indicates the likelihood of each placenta type switching trait at each node on the phylogenetic tree. We then predicted the ancestral state of the mammalian placenta by looking at the node which represents the common ancestor of mammals and the likelihood of each placental trait to be exhibited.

Results

1) Do placental traits vary with life-history traits?

Placental invasiveness

Longer lifespans (figure 1 (*i*)) and longer gestation periods (figure 2 (*i*)) were associated with a lower degree of placental invasion. In contrast, larger litter sizes were associated with a higher degree of placental invasion (figure 3(*i*)).

However, these patterns changed once phylogeny and body mass were controlled for (figures 1,2 and 3 (*ii*)). The trend for maximum longevity mostly disappeared, suggesting that relatedness between species and body mass explain most of the variation we see between maximum longevity and placental invasiveness (see table 1). For gestation length and litter size, controlling for phylogeny and body mass did not change the sign of the association with placental invasiveness. However, once phylogeny and body mass were controlled for, gestation length was the only life-history trait that significantly varied with placental invasiveness. Placentas of greatest (hemochorial) and intermediate (endotheliochorial) levels of invasiveness were associated with shorter gestation lengths, while least invasive (epitheliochorial) placentas were associated with longer gestation lengths. Therefore, for one life-history trait at least – gestation length – the observed pattern supports the prediction made by the protection hypothesis but does not support the prediction made by the cooperation/altruism hypothesis. Body mass significantly explained variation in all life-history traits, being positively associated with maximum longevity and gestation length, and negatively associated with litter size (table 1).

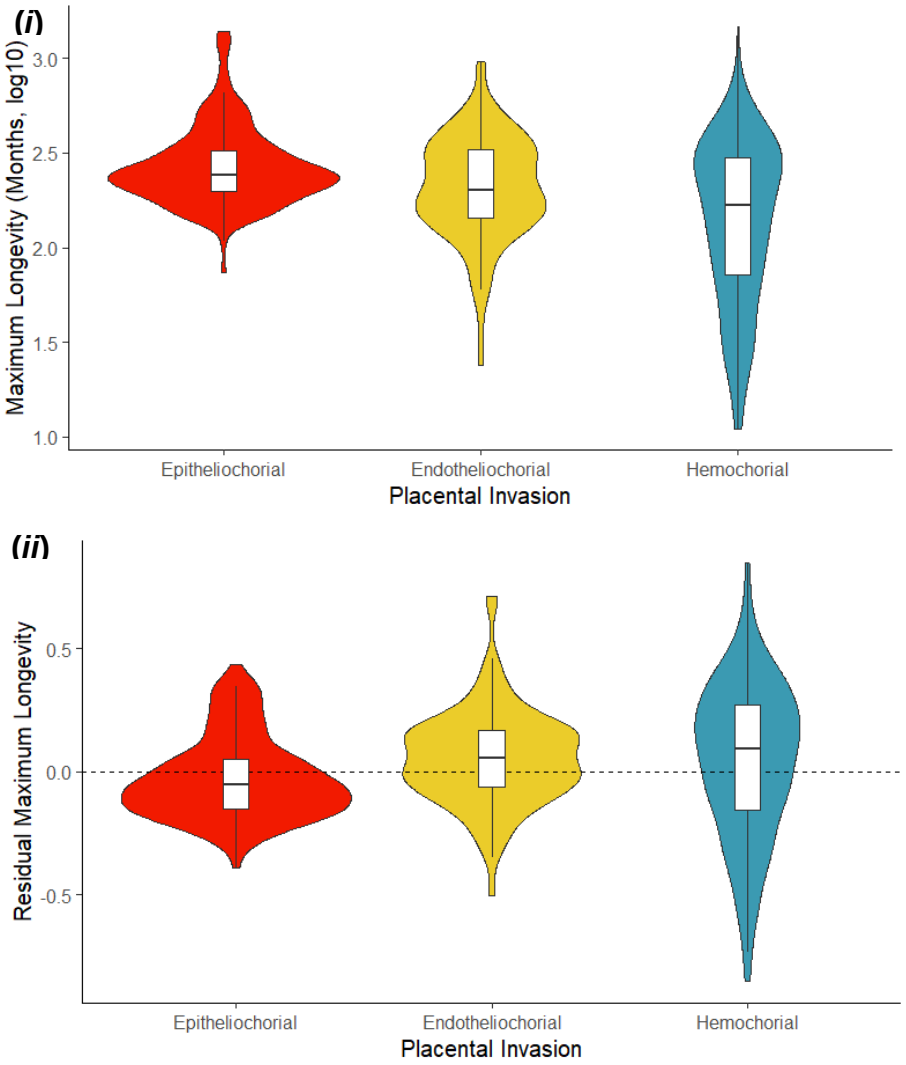

Figure 1: Maximum longevity plotted against placental invasiveness. The panel marked with an (*i*) indicates the raw relationships, whereas the panel marked with (*ii*) displays the PGLS model residual maximum longevity where body mass and phylogeny have been controlled for, this is then plotted against placental invasiveness. Red violins correspond to the least invasive epitheliochorial placenta, yellow violins correspond to the mid-invasive endotheliochorial placenta and blue violins correspond to the most invasive hemochorial placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

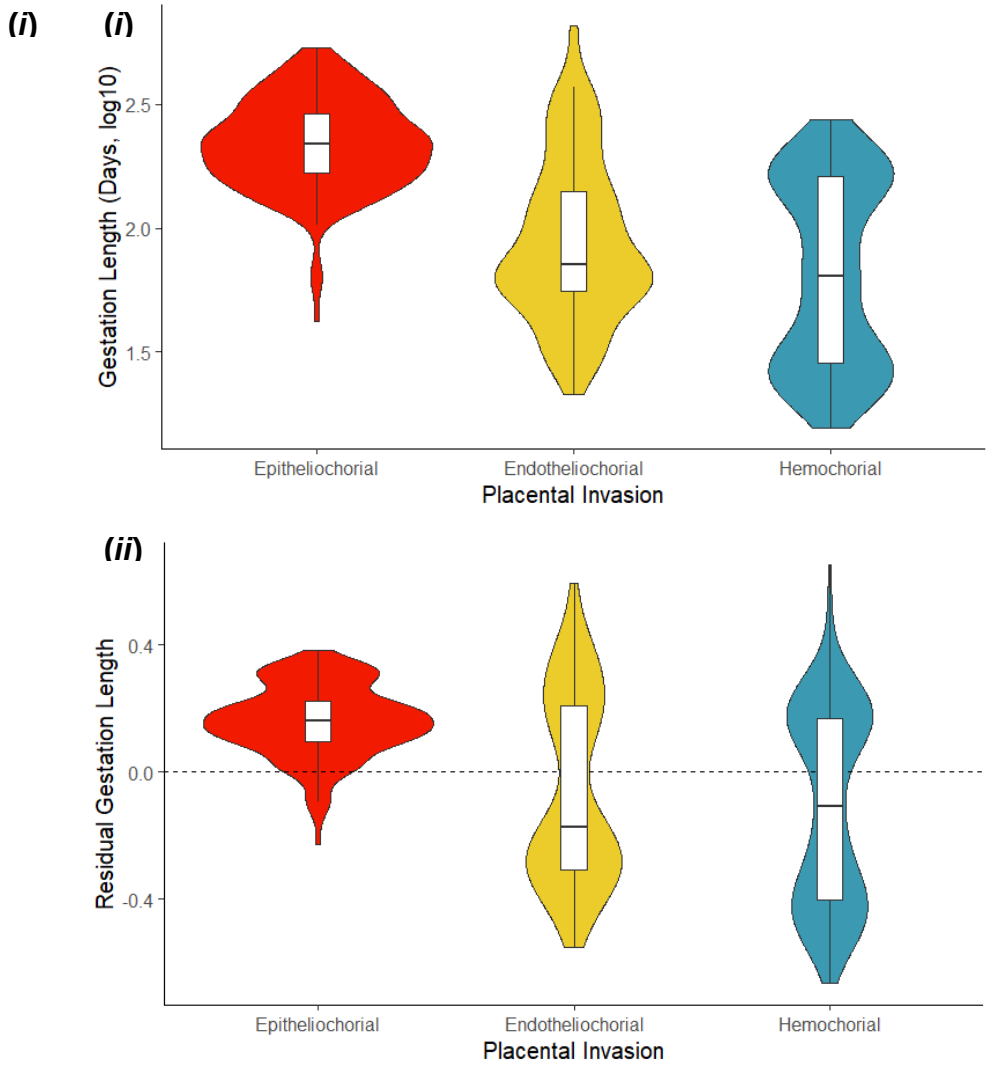

Figure 2: Gestation length plotted against placental invasiveness. The panel marked with an (*i*) indicates the raw relationships, whereas the panel marked with (*ii*) displays the PGLS model residual gestation length where body mass and phylogeny have been controlled for, this is then plotted against placental invasiveness. Red violins correspond to the least invasive epitheliochorial placenta, yellow violins correspond to the mid-invasive endotheliochorial placenta and blue violins correspond to the most invasive hemochorial placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

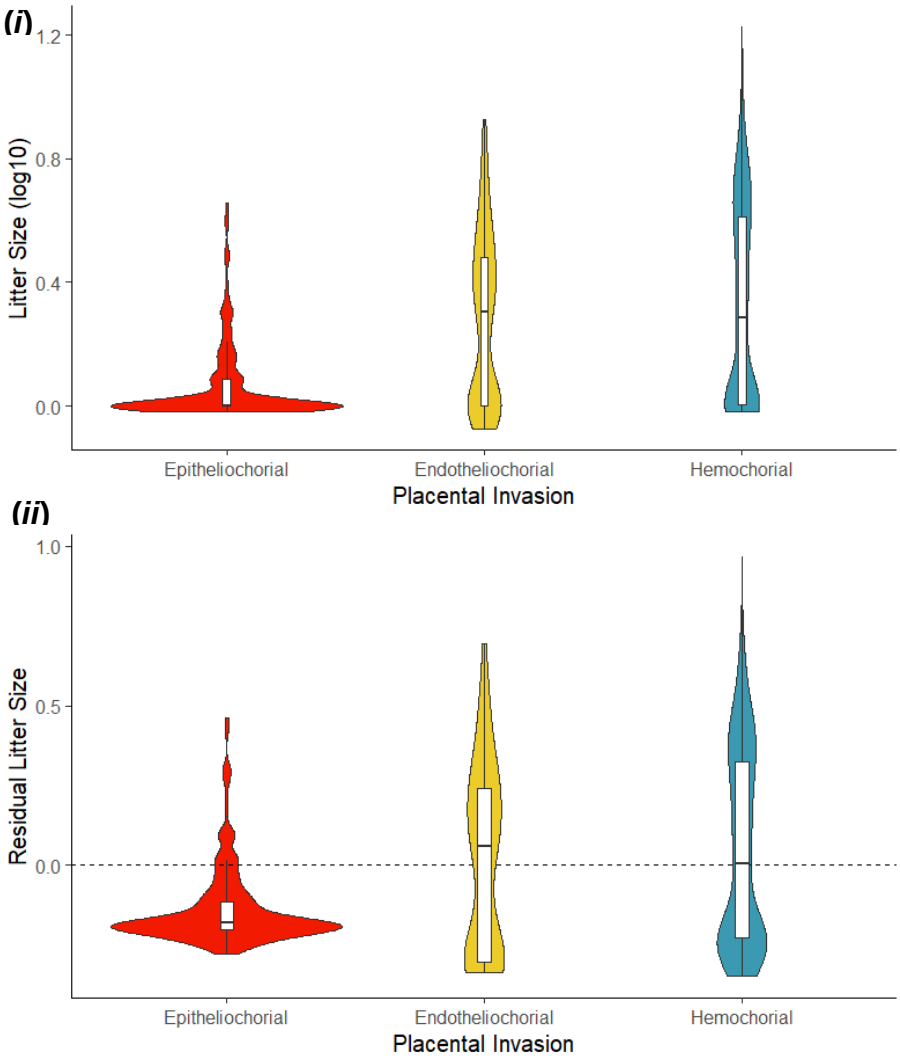

Figure 3: Litter size plotted against placental invasiveness. The panel marked with an (*i*) indicates the raw relationships, whereas the panel marked with (*ii*) displays the PGLS model residual litter size where body mass and phylogeny have been controlled for, this is then plotted against placental invasiveness. Red violins correspond to the least invasive epitheliochorial placenta, yellow violins correspond to the mid-invasive endotheliochorial placenta and blue violins correspond to the most invasive hemochorial placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

Table 1: Model estimates and standard errors for the basic linear model and the PGLS. ML corresponds to maximum longevity, GL to gestation length, LS to litter size, and BM refers to body mass. Emphasised in ***bold/italic*** are the values of interest.

				Basic Linear model		PGLS		
Life-history trait		n		Estimate+/-SE	P	Estimate+/-SE	P	λ
Without BM	ML	467	Intercept	2.426+/-0.026	<0.001	2.411+/-0.115	<0.001	0.875
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-0.108+/-0.042	0.011	-0.222+/-0.116	0.057	
			Hemochorial	-0.288+/-0.035	<0.001	-0.295+/-0.111	0.008	
	GL	567	Intercept	2.336+/-0.024	<0.001	2.290+/-0.086	<0.001	0.972
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-0.390+/-0.037	<0.001	-0.296+/-0.083	<0.001	
			Hemochorial	-0.518+/-0.031	<0.001	-0.363+/-0.081	<0.001	
	LS	660	Intercept	0.064+/-0.020	0.002	0.132+/-0.077	0.090	0.903
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	0.216+/-0.030	<0.001	0.098+/-0.075	0.193	
			Hemochorial	0.258+/-0.025	<0.001	0.165+/-0.074	0.025	
Including BM	ML	467	Intercept	1.518+/-0.051	<0.001	1.618+/-0.094	<0.001	0.697
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	0.095+/-0.033	0.004	0.015+/-0.081	0.851	
			Hemochorial	0.092+/-0.033	0.005	-0.032+/-0.077	0.675	
			Body Mass	0.193+/-0.010	<0.001	0.180+/-0.013	<0.001	
	GL	567	Intercept	1.614+/-0.046	<0.001	1.792+/-0.081	<0.001	0.942
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-0.173+/-0.033	<0.001	***-0.158+/-0.070***	***0.023***	
			Hemochorial	-0.198+/-0.031	<0.001	***-0.206+/-0.068***	***0.003***	
			Body Mass	0.156+/-0.009	<0.001	0.116+/-0.009	<0.001	
	LS	660	Intercept	0.208+/-0.044	<0.001	0.261+/-0.088	0.003	0.894
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	0.164+/-0.032	<0.001	0.060+/-0.075	0.423	
			Hemochorial	0.191+/-0.031	<0.001	0.122+/-0.074	0.098	
			Body Mass	-0.031+/-0.009	<0.001	-0.030+/-0.010	0.004	

Placental Interdigitation

Similar to the trends we observe for placental invasiveness, longer lifespans (figure 4 (*i*)*)* and longer gestation lengths (figure 5 (*i*)) were associated with a lower degree of placental interdigitation, but larger litter sizes were associated with a higher degree of placental interdigitation (figure 6 (*i*)). It is worth noting, however, that while villous and trabecular placentas did not drastically differ from one another, they both differed greatly to the labyrinthine placenta type.

These patterns changed to some extent once phylogeny and body mass were controlled for (figures 4, 5 and 6 (*ii*)). The trend for maximum longevity disappeared, suggesting that relatedness between species and body mass explained the variation we see between maximum longevity and placental interdigitation (see table 2 for details). For gestation length and litter size, controlling for phylogeny and body mass did not change the sign of the association with placental interdigitation. However, the only relationship that remained statistically significant was that between gestation length and placental invasiveness (see table 2). Higher degrees of interdigitation were associated with shorter gestation lengths (figure 5 (*ii*) and table 2). Specifically, labyrinthine placentas were associated with shorter gestation lengths than trabecular and villous placentas. It should be noted that this significant difference was only seen when comparing the villous placenta type to the labyrinthine placenta type but was not apparent when comparing villous and trabecular placentas.

Therefore, the observed pattern supports the prediction made by the protection hypothesis but does not support the prediction made by the cooperation/altruism

hypothesis. Again, all life-history traits in this analysis were significantly explained by body mass, being positively associated with maximum longevity and gestation length, and negatively associated with litter size (table 2).

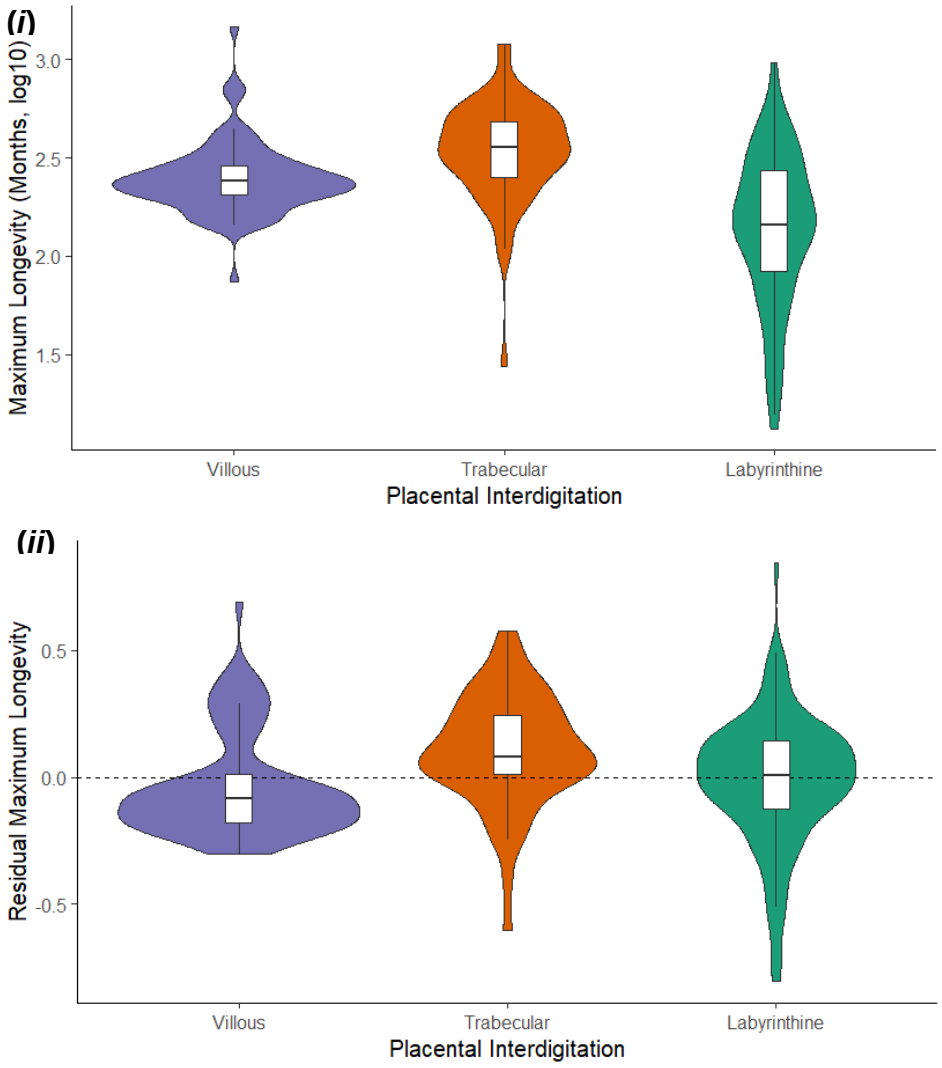

Figure 4: Maximum longevity plotted against Placental Interdigitation. The panel marked with an (*i*) indicates the raw relationships whereas the panel marked with (*ii*) displays the PGLS model residual maximum longevity where body mass and phylogeny have been controlled for, this is then plotted against placental interdigitation. Purple violins correspond to the least interdigitated villous placenta, orange violins correspond to the mid-interdigitated trabecular placenta and green violins correspond to the most interdigitated labyrinthine placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

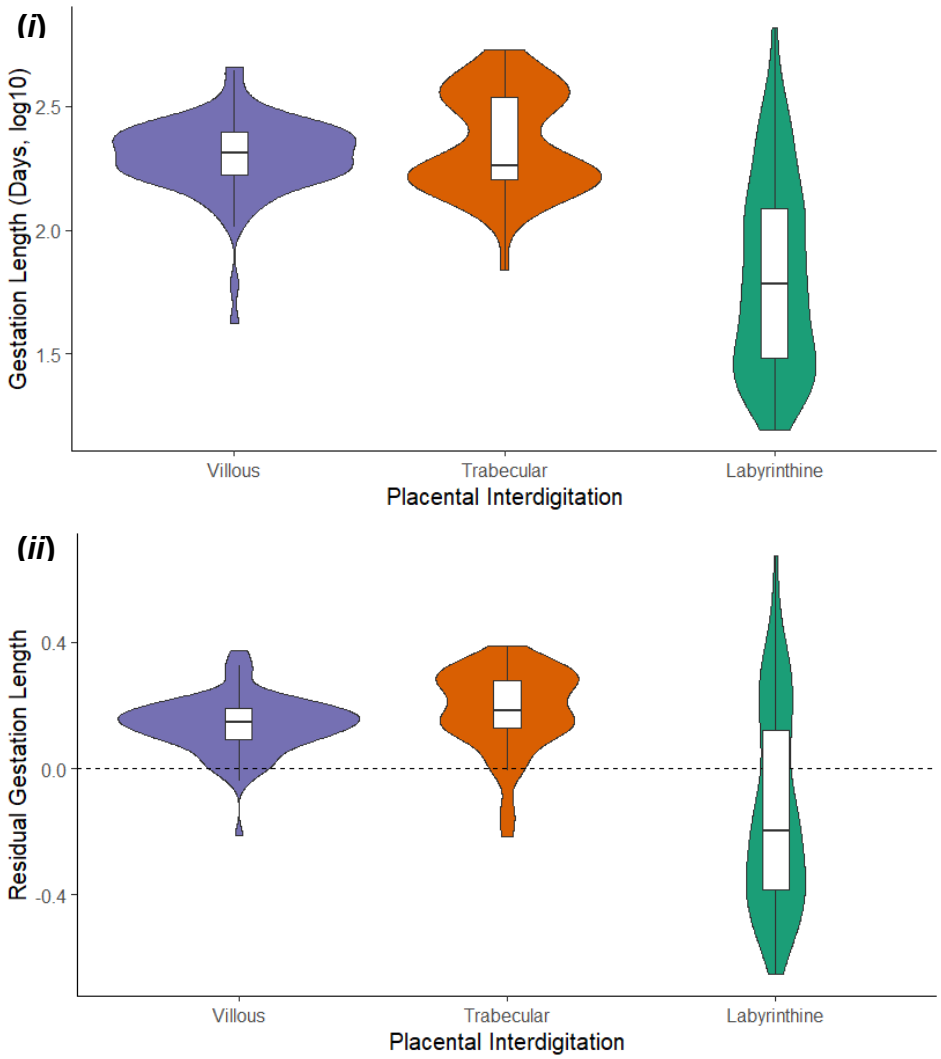

Figure 5: Gestation length plotted against Placental Interdigitation. The panel marked with an (*i*) indicates the raw relationships whereas the panel marked with (*ii*) displays the PGLS model residual gestation length where body mass and phylogeny have been controlled for, this is then plotted against placental interdigitation. Purple violins correspond to the least interdigitated villous placenta, orange violins correspond to the mid-interdigitated trabecular placenta and green violins correspond to the most interdigitated labyrinthine placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

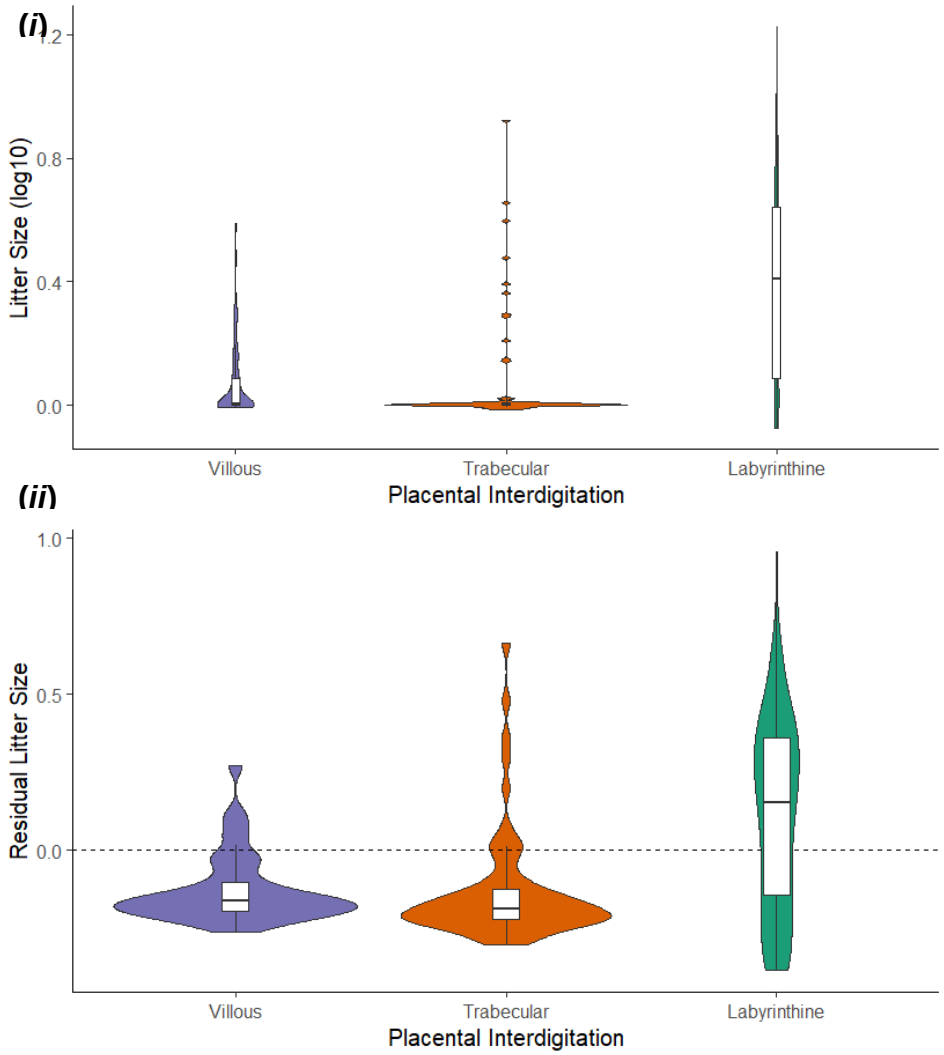

Figure 6: Litter size plotted against Placental Interdigitation. The panel marked with an (*i*) indicates the raw relationships whereas the panel marked with (*ii*) displays the PGLS model residual litter size where body mass and phylogeny have been controlled for, this is then plotted against placental interdigitation. Purple violins correspond to the least interdigitated villous placenta, orange violins correspond to the mid-interdigitated trabecular placenta and green violins correspond to the most interdigitated labyrinthine placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

Table 2: Model estimates and standard errors for the basic linear model and the PGLS. ML corresponds to maximum longevity, GL to gestation length, LS to litter size, and BM refers to body mass. Emphasized in ***bold/italic*** are the values of interest.

				Basic Linear model		PGLS		
Life-history trait		n		Estimate+/-SE	P	Estimate+/-SE	P	λ
Without BM	ML	256	Intercept	2.391+/-0.033	<0.001	2.335+/-0.115	<0.001	0.905
			Villous	-	-	-	-	
			Trabecular	0.142+/-0.052	0.007	0.030+/-0.082	0.711	
			Labyrinthine	-0.249+/-0.044	<0.001	-0.200+/-0.115	0.083	
	GL	290	Intercept	2.230+/-0.031	<0.001	2.231+/-0.083	<0.001	0.949
			Villous	-	-	-	-	
			Trabecular	0.047+/-0.050	0.341	0.012+/-0.057	0.834	
			Labyrinthine	-0.486+/-0.040	<0.001	-0.331+/-0.081	<0.001	
	LS	309	Intercept	0.069+/-0.025	0.008	0.141+/-0.084	0.092	0.971
			Villous	-	-	-	-	
			Trabecular	0.003+/-0.039	0.942	0.007+/-0.053	0.890	
			Labyrinthine	0.321+/-0.032	<0.001	0.169+/-0.080	0.035	
Including BM	ML	256	Intercept	1.665+/-0.058	<0.001	1.633+/-0.099	<0.001	0.718
			Villous	-	-	-	-	
			Trabecular	0.143+/-0.039	<0.001	-0.006+/-0.061	0.919	
			Labyrinthine	-0.009+/-0.037	0.810	-0.040+/-0.080	0.617	
			Body Mass	0.161+/-0.012	<0.001	0.182+/-0.016	<0.001	
	GL	290	Intercept	1.595+/-0.054	<0.001	1.764+/-0.082	<0.001	0.908
			Villous	-	-	-	-	
			Trabecular	0.045+/-0.037	0.230	0.002+/-0.048	0.973	
			Labyrinthine	-0.231+/-0.035	<0.001	***-0.221+/-0.067***	***0.001***	
			Body Mass	0.157+/-0.011	<0.001	0.120+/-0.012	<0.001	
	LS	309	Intercept	0.246+/-0.055	<0.001	0.303+/-0.097	0.002	0.965
			Villous	-	-	-	-	
			Trabecular	-0.001+/-0.039	0.984	0.009+/-0.052	0.868	
			Labyrinthine	0.253+/-0.036	<0.001	0.131+/-0.079	0.098	
			Body Mass	-0.040+/-0.011	<0.001	-0.043+/-0.014	0.002	

2) **What is the influence of body mass on relationships between life-history traits and placental traits?**

Placental invasiveness and placental interdigitation were found to cluster by life-history traits and by body mass (Figure 7, 8). While we expect to see strong associations between life-history traits and body mass, this analysis suggests that placenta type also predicts body mass. Table 3 shows that placental invasion significantly varies with body mass, where hemochorial placentas are associated with smaller body masses, even when controlling for phylogeny, maximum longevity, gestation length and litter size separately. If we control for phylogeny and all life-history traits together, the association remains but is only significant when comparing epitheliochorial placentas to hemochorial placentas. This suggests that body mass can be predicted by placental invasion and supports the prediction made by the protection hypothesis.

Table 4 shows that placental interdigitation significantly varies with body mass, where labyrinthine placentas are associated with smaller body masses, even when controlling for phylogeny, maximum longevity and litter size. Villous and trabecular placenta types did not significantly differ from each other, except when controlling for maximum longevity. If we control for phylogeny and gestation length, the sign does not change, but the difference in body mass between villous and labyrinthine placentas becomes non-significant. This suggests that the variation in body mass for the different levels of placental interdigitation is largely explained by gestation length. Controlling for phylogeny and all life-history traits together also renders the association between body mass and interdigitation non-significant.

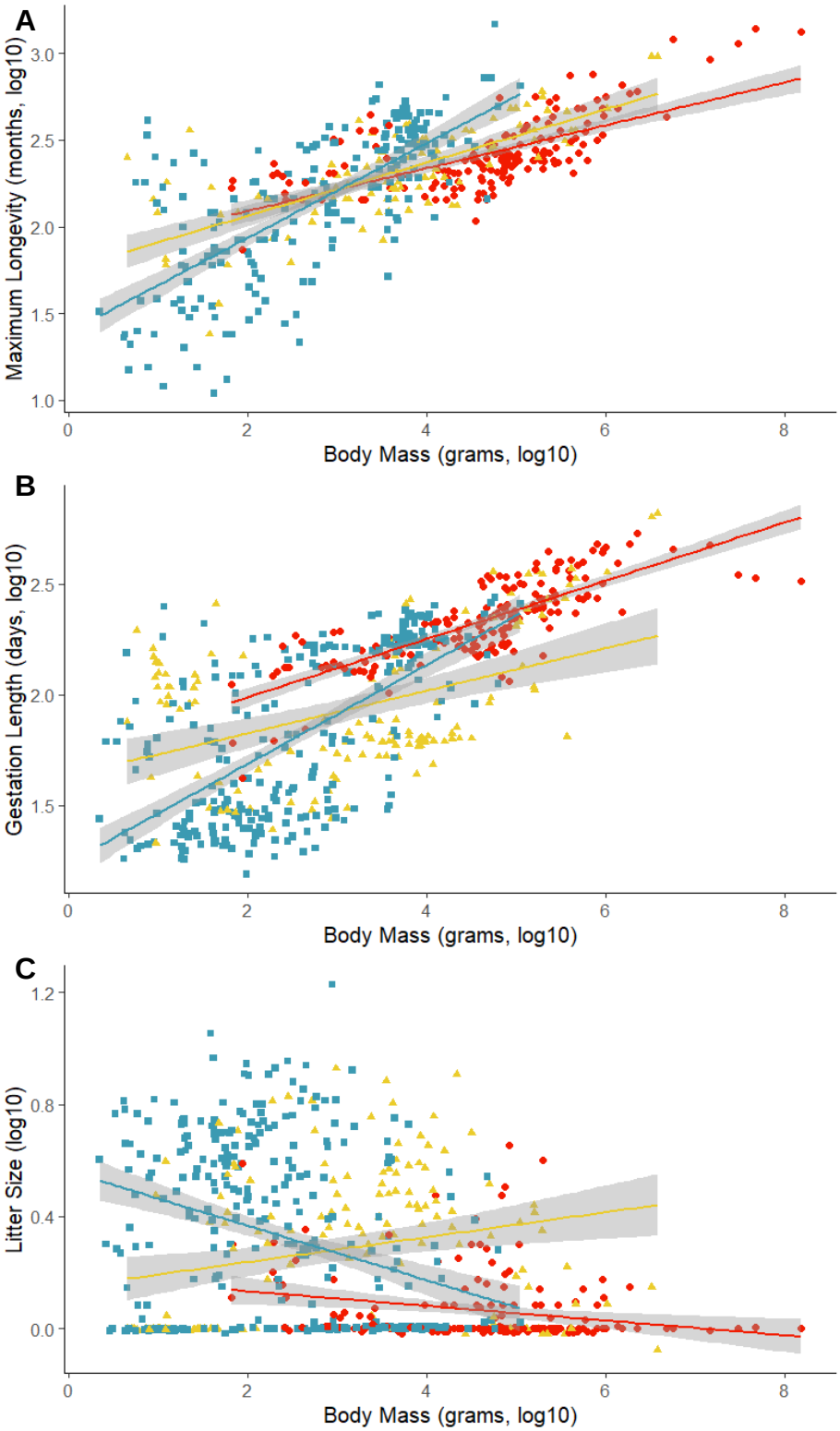

Figure 7: Relationships between placental invasiveness, life-history traits and body mass across eutherian mammal species. A) maximum longevity; B) gestation length; and C) litter size. The red line/circles correspond to the epitheliochorial placenta, the yellow line/triangles to the endotheliochorial placenta and the blue line/squares to the hemochorial placenta type. Fitted lines are lines of least squares and grey shading indicates confidence intervals.

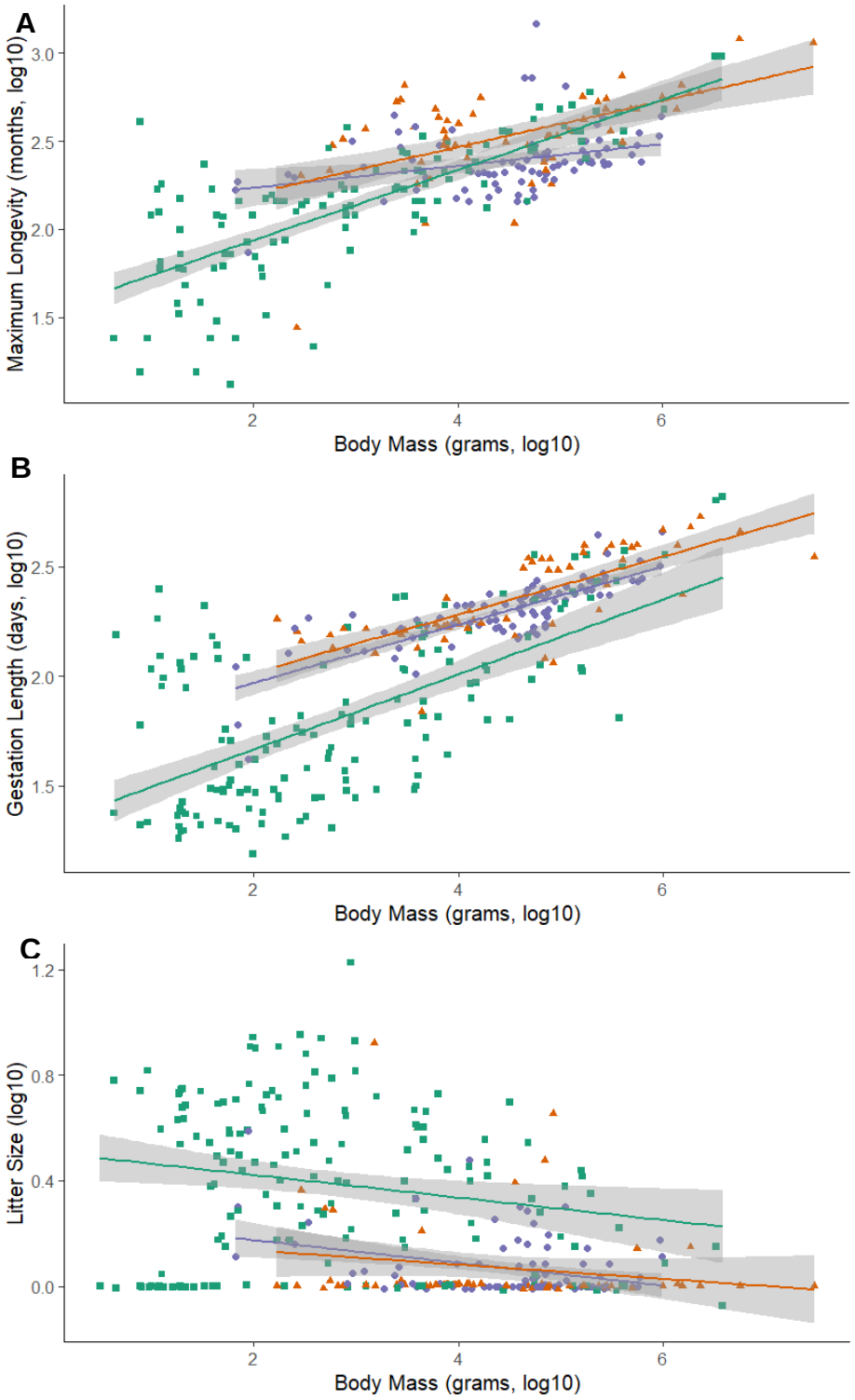

Figure 8: Relationships between placental interdigitation, life-history traits and body mass across eutherian mammal species. A) maximum longevity; B) gestation length; and C) litter size. The purple line/circles correspond to the villous placenta, the orange line/triangles to the trabecular placenta and the green line/squares to the labyrinthine placenta type. Fitted lines are lines of least squares and grey shading indicates confidence intervals.

Table 3: M**odel estimates and standard errors for the basic linear model and the PGLS** where body mass is the response variable and placental invasiveness is the explanatory variable. ML corresponds to maximum longevity, GL to gestation length, LS to litter size.

				Basic Linear model		PGLS		
	Covariate	n		Estimate+/-SE	P	Estimate+/-SE	P	λ
Without covariates	-	718	Intercept	4.664+/-0.092	<0.001	4.651+/-0.250	<0.001	0.529
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-1.923+/-0.130	<0.001	-1.523+/-0.121	<0.001	
			Hemochorial	-2.243+/-0.112	<0.001	-2.030+/-0.102	<0.001	
Including covariates	ML	467	Intercept	-0.954+/-0.299	0.002	1.998+/-0.429	<0.001	1.000
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-0.802+/-0.110	<0.001	-1.007+/-0.355	0.005	
			Hemochorial	-1.300+/-0.097	<0.001	-1.106+/-0.342	0.001	
			Maximum Longevity	2.335+/-0.120	<0.001	0.979+/-0.098	<0.001	
	GL	567	Intercept	-0.500+/-0.309	0.106	-0.428+/-0.359	0.234	0.497
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-0.537+/-0.124	<0.001	-0.406+/-0.118	<0.001	
			Hemochorial	-0.916+/-0.115	<0.001	-0.842+/-0.109	<0.001	
			Gestation length	2.200+/-0.128	<0.001	2.179+/-0.127	<0.001	
	LS	660	Intercept	4.687+/-0.092	<0.001	4.260+/-0.346	<0.001	0.997
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-1.543+/-0.139	<0.001	-1.238+/-0.332	<0.001	
			Hemochorial	-2.104+/-0.122	<0.001	-1.381+/-0.326	<0.001	
			Litter size	-0.636+/-0.175	<0.001	-0.051+/-0.120	0.669	
	ML + GL + LS	441	Intercept	-4.854+/-0.440	<0.001	-1.616+/-0.505	0.001	0.968
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-0.425+/-0.111	<0.001	-0.546+/-0.302	0.071	
			Hemochorial	-0.818+/-0.096	<0.001	-0.589+/-0.292	0.044	
			Maximum longevity	1.518+/-0.159	<0.001	0.885+/-0.104	<0.001	
			Gestation length	2.446+/-0.204	<0.001	1.659+/-0.168	<0.001	
			Litter size	1.862+/-0.217	<0.001	0.249+/-0.150	0.099	

Table 4: Model estimates and standard errors for the basic linear model and the PGLS where body mass is the response variable and placental interdigitation is the explanatory variable. ML corresponds to maximum longevity, GL to gestation length, LS to litter size. An * marks results where the standard maximum likelihood method for estimating lambda did not work and an alternative method was used (see Appendix A supplementary information for details).

				Basic Linear model		PGLS		
	Covariate	n		Estimate+/-SE	P	Estimate+/-SE	P	λ
Without covariates	-	327	Intercept	4.484+/-0.129	<0.001	3.761+/-0.348	<0.001	1.000
			Villous	-	-	-	-	
			Trabecular	-0.123+/-0.195	0.528	0.023+/-0.203	0.908	
			Labyrinthine	-1.775+/-0.160	<0.001	-0.894+/-0.331	0.007	
Including covariates	ML	256	Intercept	-1.938+/-0.474	<0.001	0.763+/-0.487*	0.119*	0.980*
			Villous	-	-	-	-	
			Trabecular	-0.392+/-0.163	0.017	0.048+/-0.218*	0.826*	
			Labyrinthine	-0.818+/-0.143	<0.001	-0.699+/-0.325*	0.032*	
			Maximum Longevity	2.696+/-0.194	<0.001	1.369+/-0.155*	<0.001*	
	GL	290	Intercept	-1.689+/-0.4.38	<0.001	-0.448+/-0.576	0.438	0.965
			Villous	-	-	-	-	
			Trabecular	-0.115+/-0.156	0.463	0.055+/-0.213	0.796	
			Labyrinthine	-0.318+/-0.155	0.041	-0.284+/-0.314	0.367	
			Gestation length	2.682+/-0.186	<0.001	1.947+/-0.217	<0.001	
	LS	309	Intercept	4.536+/-0.130	<0.001	3.864+/-0.353	<0.001	1.000
			Villous	-	-	-	-	
			Trabecular	-0.089+/-0.198	0.654	0.028+/-0.205	0.891	
			Labyrinthine	-1.374+/-0.185	<0.001	-0.772+/-0.336	0.022	
			Litter size	-1.048+/-0.288	<0.001	-0.602+/-0.222	0.007	
	ML + GL + LS	248	Intercept	-5.922+/-0.569	<0.001	-2.713+/-0.634	<0.001	0.913
			Villous	-	-	-	-	
			Trabecular	-0.354+/-0.138	0.011	0.038+/-0.192	0.841	
			Labyrinthine	-0.402+/-0.138	0.004	-0.201+/-0.279	0.471	
			Maximum longevity	1.492+/-0.209	<0.001	1.161+/-0.163	<0.001	
			Gestation length	2.920+/-0.267	<0.001	1.745+/-0.247	<0.001	
			Litter size	1.963+/-0.308	<0.001	0.320+/-0.244	0.190	

3) Do placental traits vary with mating system?

Unsurprisingly, non-monogamy was found to be more common for all placenta types, but monogamy tended to be more commonly seen in species with hemochorial and labyrinthine placentation (figure 9). In non-monogamous species, there are more epitheliochorial and villous species (low degree of invasion and interdigitation), and fewer hemochorial and labyrinthine species (high degree of invasion and interdigitation) than expected by chance (see table 5). Placental invasiveness was not independent of the mating system suggesting an association between the two (chi-square test: X^2=15.410, df=2, p<0.001), whereas placental interdigitation just misses out on a statistically significant association with mating system signifying these traits are likely independent of each other (X^2=5.929, df=2, p=0.052) (table 5).

However, further investigation using a phylogenetic logistic regression revealed that there was no statistically significant association between the mating system and any placenta type (table 6). Therefore, the prediction that larger, more invasive placentas will be associated with non-monogamous species is not supported. This indicates that the trend which appeared between placental invasiveness and mating system can be explained by phylogenetic relatedness. Furthermore, the model comparison analysis revealed that the null model, which controls for phylogeny, but contains no explanatory variables, was the best fitting model (table 7). Combinations of placental invasiveness, placental interdigitation and body mass did not explain variation in mating system better than phylogenetic relatedness alone. As no association was found between mating system and either placenta type, or any models including placenta type fit better

than the null model, mating system was not included in the comparative models below. Intriguingly, once phylogeny is considered, body mass may partially explain differences in mating system (table 6).

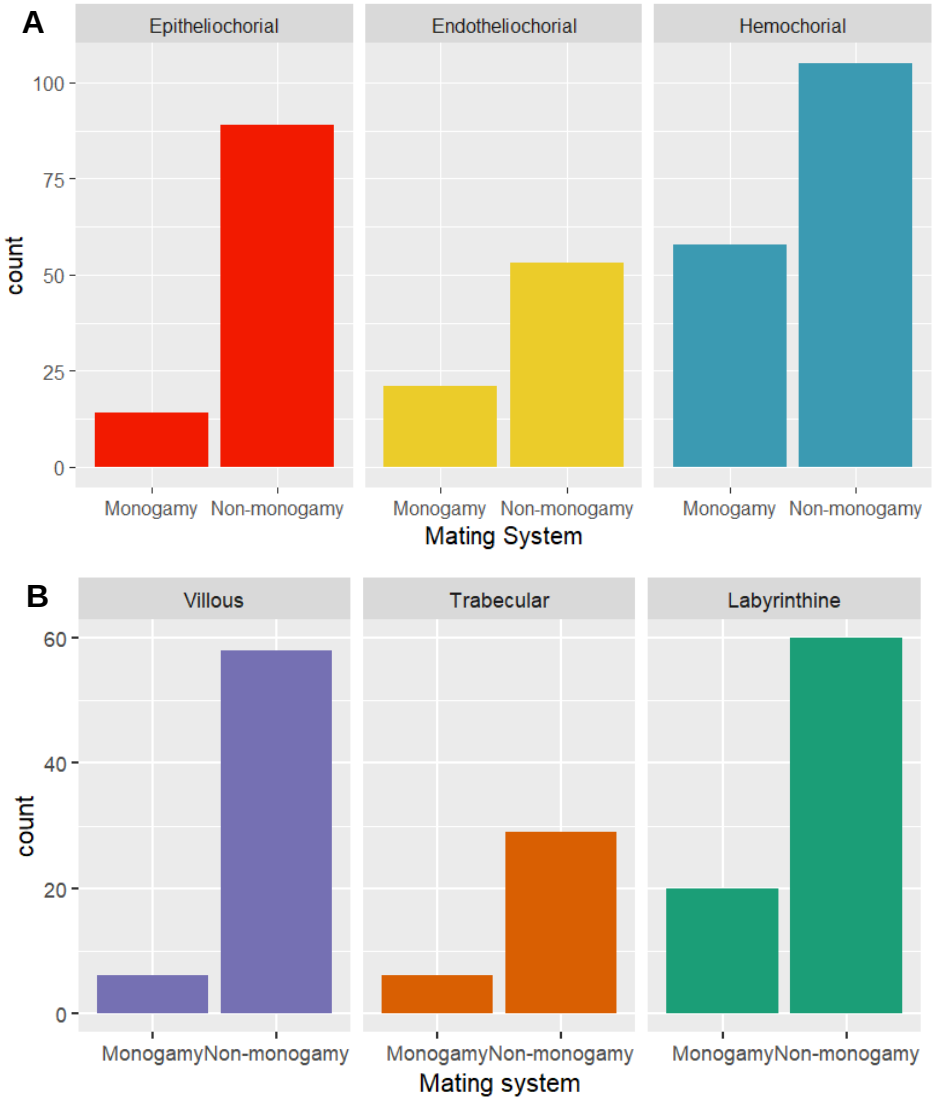

Figure 9: provides a visual representation of mating system by placental type. Panel A: Placenta interface in relation to mating system. Blue: invasive hemochorial placentation; yellow: mid-invasive endotheliochorial placentation; red: least invasive epitheliochorial placentation. Panel B: Placental interdigitation in relation to mating system. Purple: least interdigitated villous placenta; orange: mid-interdigitated trabecular placenta; green: most interdigitated labyrinthine placenta type.

Table 5: Associations between mating system, placental invasiveness and placental interdigitation. Values are observed frequencies, followed by expected frequencies in parentheses.

	Placental Invasiveness			Placental Interdigitation		
Mating System	Epitheliochorial	Endotheliochorial	Hemochorial	Villous	Trabecular	Labyrinthine
Monogamous	14(28.2)	21(20.2)	58(44.6)	6(11.4)	6(6.3)	20(14.3)
Non-monogamous	89(74.8)	53(53.8)	105(118.4)	58(52.6)	29(28.7)	60(65.7)

Table 6: Basic logistic regressions and phylogenetic logistic regression with mating system as the response variable and placental traits and body mass (BM) as explanatory variables. Mating system is binary, where monogamy =1 and non-monogamy=0. ***Bold/italic*** indicates significant results of this analysis once phylogeny and body mass have been controlled for.

				Basic logistic regression		Phylogenetic logistic regression		
Placental trait		n		Estimate+/-SE	P	Estimate+/-SE	P	α
Without BM	Placental Invasiveness	336	Intercept	1.838+/-0.288	<0.001	-1.319+/-0.908	0.147	0.010
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	-0.932+/-0.387	0.016	0.802+/-0.752	0.286	
			Hemochorial	-1.264+/-0.331	<0.001	0.909+/-0.671	0.176	
	Interdigitation	175	Intercept	-2.251+/-0.429	<0.001	-1.677+/-1.083	0.121	0.009
			Villous	-	-	-	-	
			Trabecular	0.676+/-0.621	0.276	0.503+/-0.854	0.555	
			Labyrinthine	1.204+/-0.502	0.016	1.105+/-0.815	0.175	
Including BM	Placental Invasiveness	336	Intercept	-0.199+/-0.552	0.719	0.142+/-0.913	0.876	0.018
			Epitheliochorial	-	-	-	-	
			Endotheliochorial	0.697+/-0.399	0.080	0.154+/-0.825	0.852	
			Hemochorial	0.709+/-0.369	0.054	0.367+/-0.743	0.621	
			Body Mass	-0.376+/-0.111	0.001	***-0.452+/-0.165***	***0.006***	
	Interdigitation	175	Intercept	-1.059+/-0.782	0.176	-1.137+/-1.088	0.296	0.019
			Villous	-	-	-	-	
			Trabecular	0.591+/-0.626	0.345	0.601+/-0.941	0.523	
			Labyrinthine	0.888+/-0.538	0.099	0.771+/-0.852	0.365	
			Body Mass	-0.272+/-0.152	0.074	-0.187+/-0.191	0.328	

Table 7: Model comparisons of mating system with estimates and standard errors. 'MS' refers to mating system. ***Bold/italic*** cells indicate the best fitting model.

Response variable	n	Model	AIC	α	Intercept +/- SE	Parameter estimates +/- SE				
						Invasiveness		Interdigitation		Body Mass
							Estimate +/- SE		Estimate +/- SE	Estimate +/- SE
MS	175	***Null model***	***137.554***	***0.017***	***-1.217+/-0.554***	—	—	—	—	—
		Invasiveness	150.138	0.005	-1.491+/-1.250	Epitheliochorial Endotheliochorial Hemochorial	— 0.907+/-0.803 1.140+/-0.716	—	—	—
		Interdigitation	147.622	0.009	-1.677+/-1.083	—	—	Villous Trabecular Labyrinthine	— 0.503+/-0.854 1.105+/-0.815	—
		Invasiveness + Interdigitation	156.035	0.003	-0.972+/-1.274	Epitheliochorial Endotheliochorial Hemochorial	— 0.299+/-1.091 0.573+/-0.962	Villous Trabecular Labyrinthine	— 0.526+/-0.552 0.517+/-0.999	—
		Body Mass	143.945	0.023	-0.464+/-0.658	—	—	—	—	-0.264+/-0.183
		Body Mass + Invasiveness	151.042	0.011	-1.318+/-1.245	Epitheliochorial Endotheliochorial Hemochorial	— 0.749+/-1.000 1.078+/-0.898	—	—	-0.154+/-0.191
		Body Mass + Interdigitation	148.326	0.019	-1.138+/-1.088	—	—	Villous Trabecular Labyrinthine	 0.601+/-0.941 0.771+/-0.852	-0.187+/-0.191
		Body Mass+ Invasiveness+ Interdigitation	152.260	0.011	-1.164+/-1.233	Epitheliochorial Endotheliochorial Hemochorial	— -0.641+/-1.527 -0.358+/-1.382	Villous Trabecular Labyrinthine	— 0.708+/-0.949 1.379+/-1.433	-0.103+/-0.183

4) Do placental traits explain life-history variation better than body mass alone?

Variation in maximum longevity and litter size was best explained by a null model that included only body mass as an explanatory variable. That is, placental invasiveness and interdigitation did not explain variation in these life-history traits better than body mass alone (see table 8). In contrast, for gestation length, a model with body mass and interdigitation was the best fit for the data. However, all models with placental traits and body mass explained the data slightly better than the null model (see table 8). This further supports our previous results, where gestation length was the only life-history trait that was significantly associated with placental interface and interdigitation. However, interdigitation (surface area available for exchange) coupled with body mass best explained gestation length across species.

Table 8: Model estimates and standard errors for a combination of models run on each life-history trait. ML indicates that the response variable is maximum longevity, GL gestation length and LS litter size. In all tables, 'BM' refers to body mass. ***Bold/italic*** cells indicate the best fitting model for each life-history trait.

Life-history response variable	n	Model	AIC	Intercept +/- SE	Parameter estimates +/- SE				
					Invasiveness		Interdigitation		Body mass
						Estimate +/- SE		Estimate +/- SE	Estimate +/- ST
ML	256	***BM only (null model)***	***-99.855***	1.640+/-0.109	—	—	—	—	0.170+/-0.020
		BM + Invasiveness	-95.865	1.652+/-0.165	Epitheliochorial Endotheliochorial Hemochorial	— -0.012+/-0.148 -0.013+/-0.136	—	—	0.170+/-0.021
		BM + Interdigitation	-95.986	1.655+/-0.182	—	—	Villous Trabecular Labyrinthine	— 0.018+/-0.083 -0.023+/-0.128	0.169+/-0.021
		BM + Invasiveness + Interdigitation	-92.014	1.645+/-0.170	Epitheliochorial Endotheliochorial Hemochorial	— 0.037+/-0.227 0.028+/-0.204	Villous Trabecular Labyrinthine	— 0.016+/-0.085 -0.046+/-0.197	0.169+/-0.021
GL	290	BM only (null model)	-300.780	1.647+/-0.078	—	—	—	—	0.110+/-0.014
		BM + Invasiveness	-301.595	1.825+/-0.115	Epitheliochorial Endotheliochorial Hemochorial	— -0.156+/-0.102 -0.204+/-0.094	—	—	0.104+/-0.015
		BM + Interdigitation	***-305.185***	1.829+/-0.107	—	—	Villous Trabecular Labyrinthine	— 0.004+/-0.060 -0.233+/-0.090	0.103+/-0.014
		BM + Invasiveness + Interdigitation	-302.779	1.823+/-0.118	Epitheliochorial Endotheliochorial Hemochorial	— 0.113+/-0.159 0.034+/-0.144	Villous Trabecular Labyrinthine	— 0.004+/-0.061 -0.287+/-0.140	0.101+/-0.014
LS	309	***BM only (null model)***	***-303.685***	0.395+/-0.077	—	—	—	—	-0.043+/-0.014
		BM + Invasiveness	-301.260	0.320+/-0.117	Epitheliochorial Endotheliochorial Hemochorial	— 0.038+/-0.103 0.095+/-0.096	—	—	-0.040+/-0.015
		BM + Interdigitation	-302.361	0.290+/-0.105	—	—	Villous Trabecular Labyrinthine	— 0.008+/-0.052 0.132+/-0.086	-0.039+/-0.014
		BM + Invasiveness + Interdigitation	-300.181	0.315+/-0.119	Epitheliochorial Endotheliochorial Hemochorial	— -0.143+/-0.149 -0.067+/-0.136	Villous Trabecular Labyrinthine	— 0.011+/-0.053 0.201+/-0.124	-0.038+/-0.015

5) What are the evolutionary transitions of placental traits?

Placental Invasiveness

Trait switching models revealed that the Ordered (ORD) model was the best fit for the data (AIC=199.127), above the Equal Rates (ER) model (AIC=218.635), the All Rates Different (ARD) model (AIC=203.127) and the Meristic (Mer) model (AIC=208.357) (see figure 10). Under the Ordered model, hemochorial (most invasive) placentas switched to endotheliochorial (mid-invasive), endotheliochorial placentas can switch between hemochorial or epitheliochorial (least invasive), and within this dataset, no switching occurred from epitheliochorial back to endotheliochorial, or between epitheliochorial and hemochorial.

Ancestral state reconstruction using the Ordered model for placental invasiveness indicated that the common ancestor of eutherian mammals most likely had an endotheliochorial placenta (i.e. intermediate level of invasiveness) and that placental invasiveness evolved in either direction or was retained as mammalian species radiated (figure 11). The common ancestor of ungulates likely evolved to have an epitheliochorial (least invasive) placenta, which has been retained by all ungulates alive today. Interestingly, the common ancestor of the primates likely had an endotheliochorial placenta, but then rapidly evolved in two distinct directions-lemurs, galagoes and lorises moved towards a lower degree of placental invasion like the ungulates, whereas Old World and New World monkeys and apes evolved towards a greater degree of placental invasion, and these changes likely occurred early on in their diversification. Evolution from endotheliochorial placentas to hemochorial placentas likely occurred much more

recently in Chiroptera, Afrotheriana and Xenarthra, whereas the rodents and Lagomorphs likely evolved this trait much earlier. Scandentia retain the ancestral endotheliochorial placental state along with almost all carnivores. These data indicate that trait switching can occur in either direction between endotheliochorial (moderately invasive) and hemochorial (highly invasive) placentas, but once an endotheliochorial placenta has transitioned to an epitheliochorial (least invasive) placenta, there is no evidence of it being able to switch back.

Therefore, the data do not support the prediction of the cooperative/altruistic nutrient provisioning hypothesis, namely that the ancestral state of the placenta is minimally invasive and has only evolved towards a greater degree of invasion, as our data reveal transitions away from higher invasion. Because our analysis reveals a moderately invasive placenta to be ancestral and placentas evolve both towards higher and lower levels of invasion, it is unlikely either of the other hypotheses (protection or parent-offspring conflict) are mutually exclusive. Indeed, the unidirectional transition from endotheliochorial (moderate invasion) placentas to epitheliochorial (least invasive) placentas support the prediction of the protection hypothesis, namely that placental evolution has transitioned towards a lower degree of invasion in some lineages but once this occurs switching back is not seen. The switching from endotheliochorial (moderate invasion) to hemochorial (highly invasive) placentation and back again (in some lineages this switching is seemingly frequent) is more likely to be a signature of parent-offspring conflict. Under this hypothesis, both mother and offspring are involved in a coevolutionary arms race over control of maternal resources which would theoretically drive more rapid evolution. Transitions towards greater

invasion would denote foetal control of maternal resources, whereas the transition back to moderately invasive placentation would signify mothers ‘wresting back control’ of their resources.

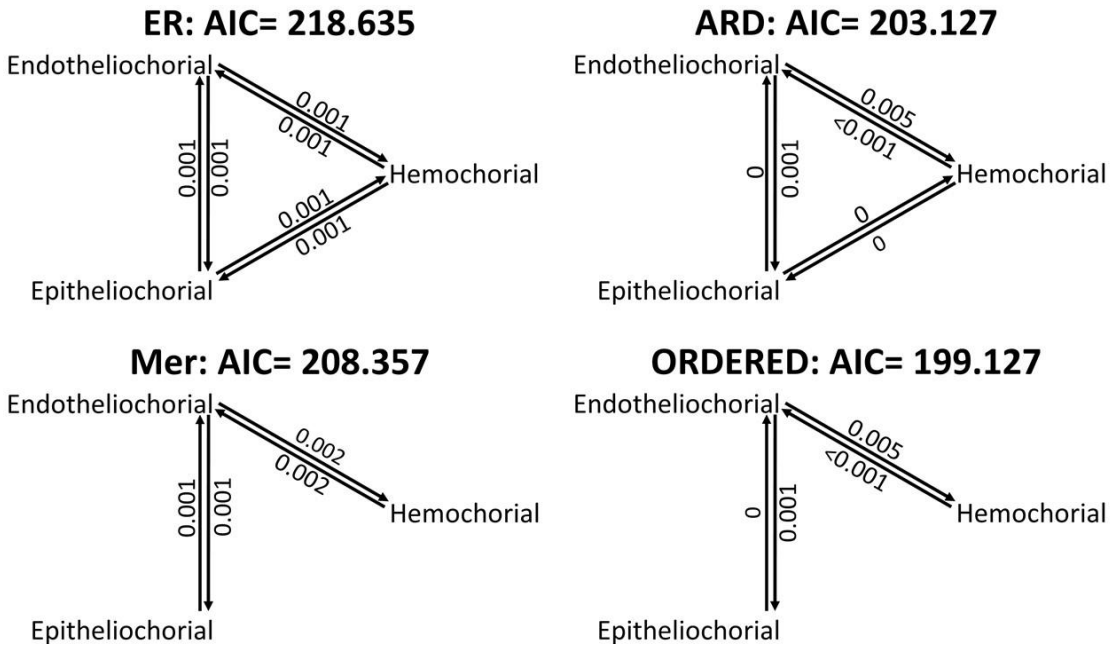

Figure 10: Schematic diagrams for the different evolutionary models of trait switching in placental invasiveness. ER represents the ‘equal rates’ model, ARD represents the ‘all rates different’ model, Mer represents the ‘meristic’ model and ORDERED represents the ‘ordered’ model. Arrows between placenta types indicate the direction of evolution, whilst the corresponding number indicates the rate at which the placenta type transition from one type to the other. Where an arrow is labelled with the rate of 0, no switching occurred between those two placenta types in that direction.

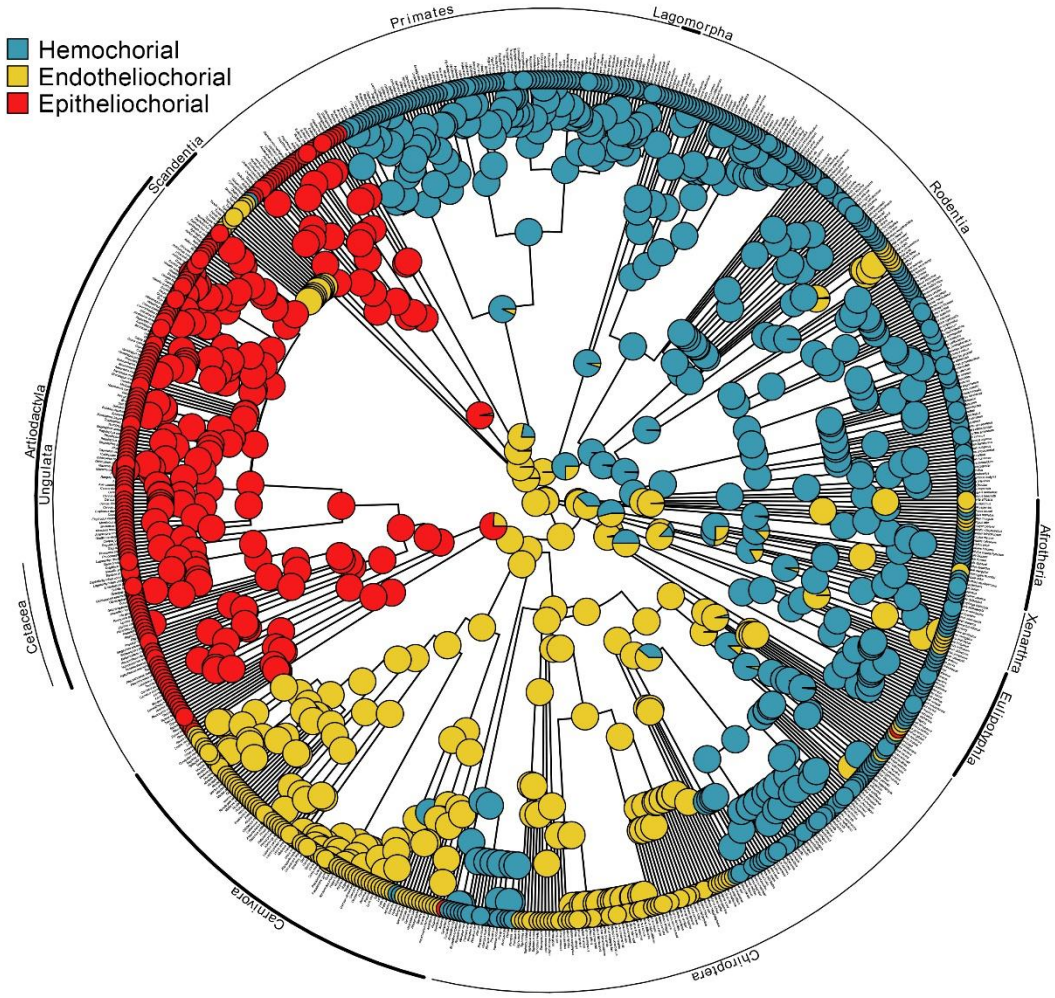

Figure 11: Ancestral state reconstruction plotted onto the mammalian phylogeny using the ORD model for placental invasiveness. The likelihood of each placental trait at each node is displayed as a mini pie chart, where red indicates the least invasive epitheliochorial placenta, yellow is the moderately invasive endotheliochorial placenta and blue is the most invasive hemochorial placenta. The node at the centre represents the common ancestor of eutherian mammals, and nodes at the outer edge represent the extant species labelled in the phylogeny. Eutherian mammalian clades are labelled around the outside of the tree.

Placental Interdigitation

Trait switching models for placental interdigitation revealed that the Ordered model best described the data (AIC=132.703) when compared to the ER model (AIC=143.087), the ARD model (AIC=136.285) and the Mer model (AIC=138.153) (see figure 12). Labyrinthine placentas (greatest surface area available for exchange) switched to trabecular placentas and trabecular placentas switched to villous placentas (smallest surface area for exchange), but no switching occurred between labyrinthine and villous placenta types. Interestingly, these evolutionary transitions suggest that evolution towards a greater degree of interdigitation has not occurred.

The ancestral state reconstruction for interdigitation using the ordered model indicates that the common ancestor of mammals likely had a labyrinthine placenta, and species either retained this trait or evolved placentas with smaller surface areas (figure 13). The common ancestor of Primates and the common ancestor of Ungulates rapidly evolved lower degrees of interdigitation, whereas, in the clade Xenarthra, a lower degree of interdigitation evolved much later, and thus is not displayed across the whole clade. Most Lagomorphs, Rodents, Afrotherians, Eulipotyphla, Chiropterans, Carnivores and Scandentians retain the ancestral labyrinthine state. It seems likely that trait switching in placental interdigitation has only ever moved towards a lower degree of interdigitation and has never switched back.

These results for the trait switching and ancestral state reconstruction of placental interdigitation supports the prediction of the protection hypothesis, i.e. that

placental evolution has transitioned towards a lower degree of interdigitation in some lineages. This is likely attributed to the oxidative shielding hypothesis, as placental interdigitation is thought to have little bearing on pathogen transmission across the placenta. This result could also provide some support for parent-offspring conflict whereby mothers wrest back control of their resources, but the lack of rapid switching back and forth between any two placenta types suggests that perhaps foetuses do not have any significant influence over maternal resources via changes to interdigitation and is therefore unlikely. However, the data do not support the prediction of the cooperative/altruistic nutrient provisioning hypothesis, that the ancestral state of the placenta is minimally interdigitated and has evolved towards a greater surface area.

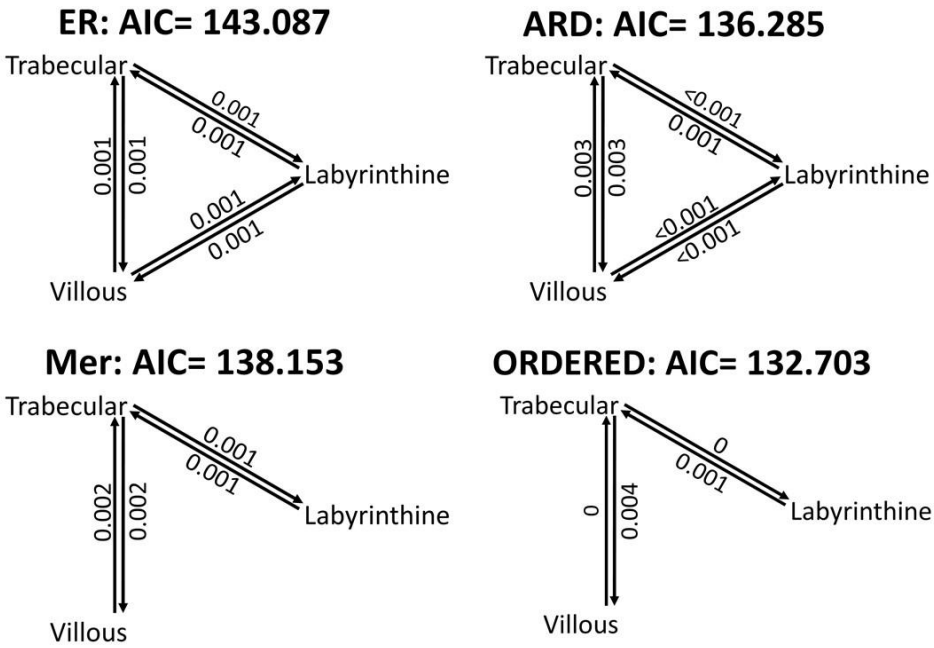

Figure 12: Schematic diagrams for the different evolutionary models of trait switching in placental interdigitation. ER represents the 'equal rates' model, ARD represents the 'all rates different' model, Mer represents the 'meristic' model and ORDERED represents the 'ordered' model. Arrows between placenta types indicate the direction of evolution, whilst the corresponding number indicates the rate at which the placenta type transition from one type to the other. Where an arrow is labelled with the rate of 0, no switching occurred between those two placenta types in that direction.

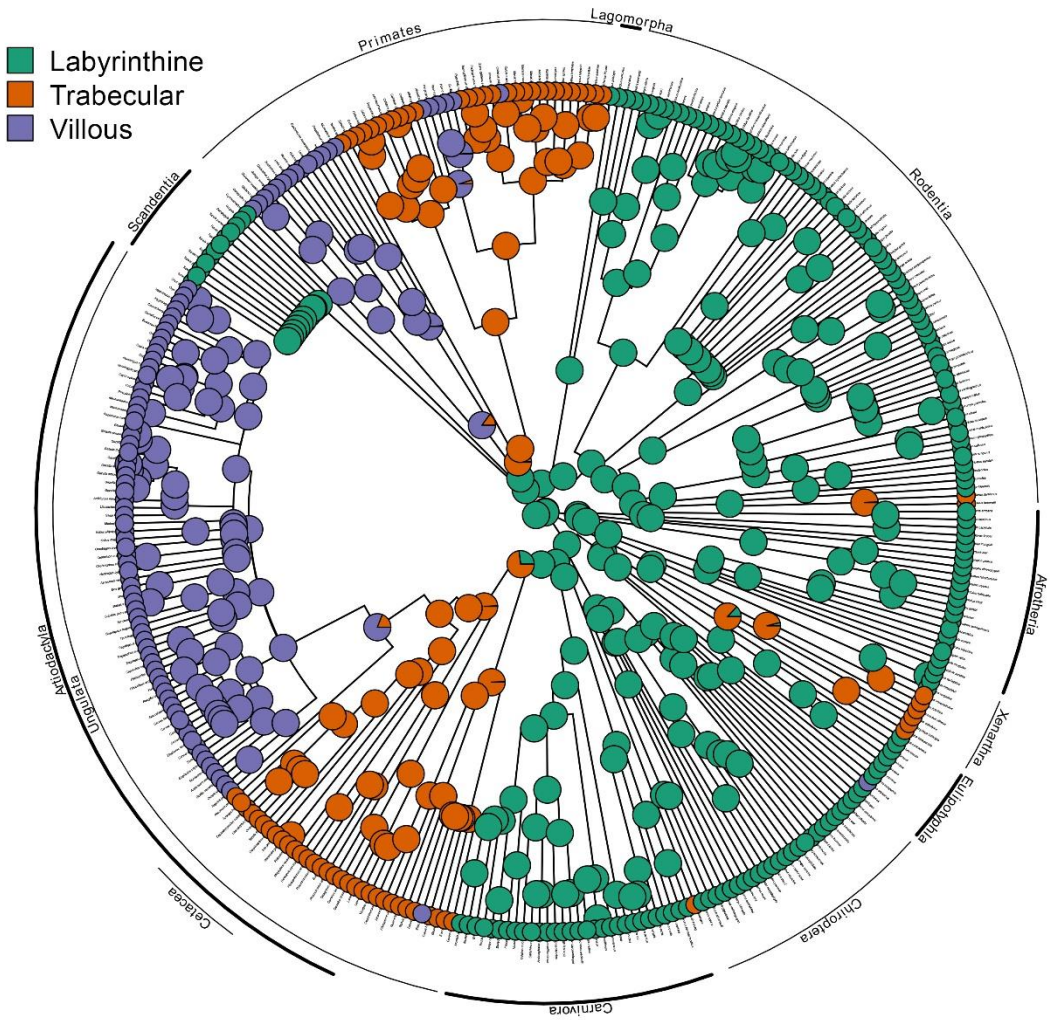

Figure 13: Ancestral state reconstruction plotted onto the mammalian phylogeny using the ORD model for placental interdigitation. The likelihood of each placental trait at each node is displayed as a mini pie chart, where purple indicates the villous placenta lowest degree of interdigitation, orange is the trabecular placenta and green is the labyrinthine placenta with the highest degree of interdigitation. The node at the centre represents the common ancestor of eutherian mammals, and nodes at the edge represent the extant species labelled in the phylogeny. Eutherian mammalian clades are labelled around the outside of the tree.

Discussion

This study has shown that all life-history traits that were examined varied significantly with placental interface and interdigitation if body mass and phylogeny were not statistically controlled for. Hemochorial, endotheliochorial and labyrinthine placentation were associated with shorter lifespans, shorter gestation lengths and larger litter sizes. Gestation length was the only life-history trait that was associated with both placental interface and interdigitation once body mass and phylogeny were controlled for, where shorter gestation lengths were still associated with hemochorial and endotheliochorial invasiveness and labyrinthine interdigitation (tables 3 and 4). The key variable that explained gestation length was placental interdigitation when coupled with body mass (phylogenetically controlled), but including placental interface also explained the data better than body mass alone. Both variation in maximum longevity and litter size was best explained by body mass coupled with relatedness between species and were not associated with any placenta types (see table 8). Body mass varied significantly with both placental invasiveness and interdigitation. For invasiveness, there was a difference between (at least some categories of) placenta type and body mass even when controlling for all life-history variables and phylogeny, where smaller body mass is associated with a higher degree of invasion. For interdigitation, the pattern is broadly the same except there was no association when controlling for gestation length. This clustering of placenta type by body mass provides some support for our metabolic scaling argument. The results of the trait switching analysis and ancestral state reconstruction revealed that both placental interface and placental interdigitation followed an ordered evolutionary progression and that the ancestral state of the mammalian placenta was most likely to be labyrinthine and endotheliochorial.

In contrast to previous studies which found associations between the pace of life and placental traits (Pires, McBride and Reznick, 2007; Lewitus and Soligo, 2011; Pires *et al.*, 2011; Garratt, Gaillard, *et al.*, 2013), we did not find a direct link between maximum longevity or litter size with either placental invasiveness or interdigitation. Garratt, Gaillard, *et al*.'s (2013) study found that maximum longevity and offspring per year (similar to our litter size variable) varied with the placental interface, interdigitation or both (Garratt, Gaillard, *et al.*, 2013). This led to the conclusion that evolutionary changes in life-history traits could be driving the evolution of different placenta types as a consequence of parent-offspring conflict. This has previously been called the 'life-history facilitation hypothesis' (Pires *et al.*, 2011) and would explain why we see the grouping of particular traits across mammals (Lewitus and Soligo, 2011). Our results seemingly contradict this; our much larger data set reveals that only gestation length can be associated with placenta invasion and interdigitation, where shorter gestation lengths were associated with higher degrees of invasiveness and interdigitation (although no difference was found between the villous and trabecular interdigitation). Body mass and phylogenetic relatedness explained the raw trends we observed with the other life-history variables. While we did not find significant associations with all life-history variables, our finding that higher placental invasion and interdigitation were associated with shorter gestation lengths provides support for the protection hypothesis, over the cooperative/altruism hypothesis which predicts less invasive, less interdigitated placentas to be associated with a faster pace of life.

We also found that placental interdigitation was the key trait that explained gestation length- this has previously been predicted by Wildman *et al.*, (2006)

and observed by Capellini, Venditti and Barton, (2011). Our study also revealed that placental invasiveness still had some bearing on gestation length, a more intimate connection between maternal and fetal blood also seemed to be associated with decreased gestation length. In theory, it makes perfect sense that gestation length is associated with placenta type, it would be surprising if we did not find this. Gestation length is the only life-history trait we tested that directly links to the placenta; the length of time a foetus spends growing will be directly linked to the rate of maternal provisioning which can only be mediated by the placental interface. It has been previously noted that placental surface area has a significant bearing on gestation lengths. Starck pointed out that the epitheliochorial placenta of a cow must be more efficient than the haemochorial human placenta; with a similar gestation period of approximately 9 months, a newborn human is only 3.2kg and altricial, whereas a new-born calf is 35kg and precocial (Starck, 1959). These seemingly counterintuitive moves away from more intimate invasion should likely be made up for in other ways, evolving other very different placental structures which have not been addressed in the scope of this paper, may compensate for this apparently detrimental evolution. For example, the full-term human placenta is a discoid shape and has a surface area of approximately $15m^2$ whereas the cow's epitheliochorial placenta has a cotyledonary shape and has a surface area of $120m^2$ (Baur, 1981; Vogel, 2005). Indeed, interdigitation influences the total surface area available for exchange. This can explain why moderately invasive endotheliochorial placentas (which are exclusively labyrinthine in our dataset) display similar gestation lengths to hemochorial placentas (which may have any form of interdigitation) if only invasiveness is taken into account (Capellini, Venditti and Barton, 2011). Wildman suggests that villous interdigitation could entail less of a metabolic

demand on the mother than labyrinthine interdigitation, allowing her to sustain longer gestation periods (Wildman *et al.*, 2006). This could be a symptom of parent-offspring conflict allowing mothers greater control over their resources. However, if interdigitation does influence metabolic demand as Wildman *et al.* suggest, the allometric scaling of WSMR could mean that a higher degree of interdigitation can facilitate the higher metabolic rate of smaller species. This in turn may put these species at higher risk from intergenerational oxidative stress and consequently have some bearing on life-history evolution.

We found a strong association between body mass and placental invasiveness, even when controlling for phylogenetic relatedness and other life-history variables. Associations between body mass and placental interdigitation are largely the same, except this trend disappears when gestation length is controlled for. It has been previously been noted that placental invasiveness associates with body mass (Elliot and Crespi, 2008, 2009; Martin, 2008), but this is the first time it has been found to associate with interdigitation too. We see that heavier species are likely to exhibit a villous/trabecular or epitheliochorial placentation, and species with a small body mass are likely to display hemochorial or labyrinthine placentation. However, it seems that gestation length explains why greater interdigitation is associated with smaller body mass, this fits neatly with our other results that point to interdigitation and gestation length coevolving. We know that body mass predicts life-history traits (Western, 1979; Peters, 1983; Schmidt-Nielsen, 1983; Dobson and Oli, 2007) and can now confirm that placental form predicts body mass. However fascinatingly it seems that, with the exception of gestation length, placental traits do not directly predict life-history traits . This is perhaps a consequence of life-history traits being subject to a

variety of environmental factors, for example, temperature, resource availability, predator-prey dynamics etc., this phenomenon is known as phenotypic plasticity (Stearns, 1992; Roff, 1997; Pigliucci, 2001; Dewitt and Scheiner, 2004). The fluid nature of life-history dynamics may provide a chance for these traits to rapidly evolve even when they are largely facilitated by body mass. Contrastingly, placental traits lack this fluid nature and are presumably bound by genetic mutations, these mutations must randomly arise and prove advantageous to be selected for and spread throughout the population, thus cannot evolve so rapidly. This may explain why we do not see life-history traits directly associating with specific placenta types even while they both strongly associate with body mass.

The association between body mass and placentation leaves room for debate about Pires' *et al*'s life-history facilitation hypothesis (Pires *et al.*, 2011). Having found no association between placentation and maximum longevity (a measure of senescence) or litter size (a measure of fecundity), it is tempting to conclude that we find no evidence to support life-history facilitation. However, the apparent strong association with body mass, which is known to predict life-history traits (Western, 1979; Peters, 1983; Schmidt-Nielsen, 1983; Dobson and Oli, 2007), calls this conclusion into question. Our proposed metabolic scaling idea may offer a solution to this paradox. If WSMR is key to determining placentation, then we should find that increasing placental invasion or interdigitation, which is associated with smaller species, is better suited to the facilitation of their higher WSMR compared to larger species. Indeed, foetuses *in utero* exhibit an adult-like specific metabolic rate rather than a higher metabolic rate predicted by their foetal body mass, essentially acting as an extension of the maternal phenotype. This is thought to be due to the limited oxygen transport capacity of the placenta (Rahn,

1982; Paganelli and Rahn, 1984; Singer and Mühlfeld, 2007), offspring only reach their expected higher metabolic rate according to their body weight after birth (Bohr, 1900; Hasselbalch, 1900; Wilkie, 1977; Wieser, 1984; Singer and Mühlfeld, 2007). If more intimate forms of placentation increase the oxygen transport capacity of the placenta, this could facilitate the higher metabolic demand of foetuses of smaller species *in utero*, and thus explain why we observe smaller species exhibiting higher levels of invasion and interdigitation. However, theoretically, this comes with a greater risk of intergenerational oxidative stress, less investment in damage reduction could select for shorter lifespans and a faster pace of life. Indeed, maternal oxidative stress has been found to negatively influence offspring production, development and survival (Bize *et al.*, 2008; Møller, Karadas and Mousseau, 2008; Essa *et al.*, 2015; Vitikainen *et al.*, 2016; Dupoué *et al.*, 2020), which has the potential to influence life-history evolution. Under this logic, the association between placentation and body mass supports the life-history facilitation hypothesis, if intergenerational oxidative stress does play a significant role. However, this would require further empirical testing of associations between placentation and metabolism.

Although we predicted that monogamy would be associated with a lower degree of placental interdigitation and placental invasion, we found no association between mating system and placenta type, nor did any model containing placenta types fit better than the null model. Therefore, mating system was not included in the comparative models. Curiously, there does seem to be an association between mating system and body mass in the model with placental interface, where species with larger body masses tend towards non-monogamy, although it is unclear why. Non-monogamy is by far a more common strategy in nature.

The evolution of mating systems is thought to be driven by a variety of ecological and phylogenetic factors, where the system which evolves depends on which sex is limiting and the degree to which the limited sex can monopolize mates, control a resource base or both (Emlen and Oring, 1977; Wittenberger, 1979). It was novel speculation that mating system would vary with placenta type as a consequence of genomic imprinting via the parent-offspring conflict, however, this result suggests that particular mating systems do not drive the evolution of placental types or *vice versa*. Consequently, mating system likely has a limited influence on parent-offspring conflict from a placental intimacy perspective. However, it is possible that mating system could vary with a different aspect of placentation, but this would also need further empirical testing by exploring associations between mating systems and other placental traits, such as placental shape, placenta-foetus ratio, capillary position, or interactions between them.

It is unclear why our results differ somewhat from those of previous studies. The discrepancy in results for maximum longevity could partially be explained by the use of different datasets for a very similar analysis. Garratt *et al* (2013) used the AnAge dataset (De MagalhÃes and Costa, 2009) which incorporates data from databases, journal articles, books, websites, and personal communications. In contrast, we opted to use the longevity records in PanTHERIA (Jones *et al.*, 2009) which are taken from primary, secondary or extrapolated literature sources. We also used different fecundity measures; while we used litter size, Garratt, Gaillard, *et al* (2013), used the number of offspring per year, also taken from the PanTHERIA dataset and calculated by multiplying average litter size and number of litters per year. Interestingly, the same gestation length data were used for the

present study and by Garratt, Gaillard, *et al*. (2013); in both cases, there was a significant association between gestation length and interdigitation. However, Garratt, Gaillard, *et al.* (2013) found that transitions away from invasive placentation were associated with an increase in the pace of life, which contradicts earlier findings (Lewitus and Soligo, 2011) and our results. This difference could be because Garratt, Gaillard *et al*. (2013) allowed for the comparison of the effects of placental interface and interdigitation in the same model, whereas Lewitus and Soligo (2011a) and the present study examined the effects of placental invasion and interdigitation separately.

Our ancestral state reconstruction, the largest of its kind, reveals that the first eutherian placenta was most likely endotheliochorial or moderately invasive, and labyrinthine or highly interdigitated. This agrees with several previous studies that also conclude that intermediate invasion (Carter and Enders, 2004; Mess and Carter, 2006; Martin, 2008) and labyrinthine interdigitation (Wildman *et al.*, 2006) is ancestral. With more than double the sample size of the next largest study, we disagree with those who find hemochorial invasion to be the ancestral state (Elliot and Crespi, 2006, 2009; Wildman *et al.*, 2006). This is an exciting finding as it suggests that species radiated out in different evolutionary directions under different selection pressures rather than in a linear fashion. The best-fitting model of placental invasion revealed an ordered evolutionary progression, transitions towards lower epitheliochorial invasion and could indicate placental protection in some lineages or a transition towards maternal control over resources. However, in all mammalian lineages that evolve epitheliochorial placentation, there is no evidence that they have ever switched back. This suggests that an evolutionary arms race over a conflict of interests (e.g. parent-offspring conflict) is an unlikely

cause. Contrastingly, evolutionary transitions towards greater invasion could indicate maternal-foetal cooperation/altruism or a transition towards foetal control of maternal resources. However, we see transitions both back and forth between endotheliochorial and hemochorial invasion. Coupled with the lack of evidence for cooperative altruism in the analysis of trait covariation, we might conclude that parent-offspring conflict remains a better explanation for these evolutionary transitions. Our finding that placental interdigitation also follows an ordered evolutionary progression and can only transition towards a lower degree of interdigitation, provides a compelling argument for a protective function. Overall, these results suggest that the evolution of placental invasion and interdigitation is not driven by increasing efficiency of nutrient transfer from mother to foetus. Species that transition to a lower degree of interdigitation are essentially lowering the surface area available for nutrient/waste exchange, which would theoretically reduce transfer efficiency. However, this is probably an overly simplistic view (Wooding and Burton, 2008). Reduced interdigitation may likely be compensated at least in part, by changes in placental shape, size, capillary position, regional specialisation or even a transition towards greater invasion. This warrants further study.

This study has not been able to fully untangle the role of placental protection of intergenerational oxidative stress and vertical transmission of pathogens from maternal blood (Loke, 1982; Webster and Kapel, 2005; Capellini, Nunn and Barton, 2015). The evolutionary transitions away from high placental invasion, in particular, could equally be explained by the oxidative shielding hypothesis and by protection against vertical transmission of pathogens. Placental invasion is likely the only placental trait relevant to vertical transmission of pathogens, as the

intimacy between maternal and foetal blood should have a bearing on what can or cannot cross the placental barrier, but interdigitation is predicted to influence the rate of vertical transmission of substances. Finding transitions away from greater degrees of interdigitation could be attributed to oxidative shielding as a possible explanation, but still cannot ultimately rule out the role of pathogen protection. Overall, this does provide further support for a protective function of the placenta, but cannot fully explain the move towards greater placental invasion in some lineages. A potential explanation for transitions towards greater invasion in some lineages is the contradictory idea that the foetus may gain greater protection via increased placental invasion. This takes the form of greater transmission of antibodies across the placenta to develop the foetal immune system prior to birth (Baintner, 2007; Chucri *et al.*, 2010). Indeed, higher placental invasion allows maternal antibodies to pass readily from mother to foetus, yet maternal antibodies are effectively non-existent in foetuses of species with non-invasive epitheliochorial placentation (Baintner, 2007; Wooding and Burton, 2008; Chucri *et al.*, 2010). However, it remains unclear why this would be a necessary adaptation for r-selected species which are defined by lower parental investment, especially when maternal antibodies can also be passed down to offspring via colostrum after birth (Chucri *et al.*, 2010). Capellini, Nunn and Barton (2015) provide a counter-argument to this, having found that while bacterial species richness is higher in species with low placental invasiveness (suggesting a protective placental function), protozoa species-richness is highest for invasive hemochorial placentation (Capellini, Nunn and Barton, 2015). This could explain why greater foetal immunity is necessary for hemochorial species prior to birth, but may also be a secondary adaptation in response to evolutionary selective

pressures that drive the evolution towards greater placental invasiveness (Capellini, Nunn and Barton, 2015).

These findings open many potential avenues for future research. Unfortunately, due to the current limitations of phylogenetic analytical techniques, we were unable to run models using placenta type as the response variable (Capellini, 2019; Stott, 2019). However, if this were possible in future, and body mass was found to be the best predictor of placenta type, it would be important to explore the physiological mechanism that would underpin this finding. Although it continues to remain an enigma, metabolic scaling may explain why body mass both governs the evolution of life-history traits and associates strongly with placentation; establishing whether oxidative stress plays a role in this mechanism could be crucial to develop our understanding of life-history evolution. This study also highlights the importance of evaluating the use of body mass as a covariate in comparative life-history analyses. Broadening the range of life-history and placental traits used and exploring interactions between them could be useful for testing ideas about whether differences in placentation could be a consequence of allometric scaling and provide a more comprehensive understanding of the factors driving placental evolution.

In conclusion, our study seemingly contradicts the well-established idea that placentation facilitates life-history evolution (Pires, McBride and Reznick, 2007; Lewitus and Soligo, 2011; Pires *et al.*, 2011; Garratt, Gaillard, *et al.*, 2013), but shows that gestation length is the main life-history trait associated with placentation. However, the surprisingly strong association between placentation

and body mass requires further investigation. It is possible that this is a symptom of the allometric scaling of weight-specific metabolic rate, which could indirectly affect species' life-histories via the intergenerational transmission of oxidative stress. This highlights the importance of evaluating whether body mass should be considered as more than just a covariate in comparative life-history studies and provides further motivation to understand the problem of scaling. We find very little convincing evidence that placental invasion or interdigitation evolve according to the cooperative/altruism hypothesis, but transitions away from greater placental invasion and interdigitation support the idea of the placenta playing a protective role. Coupled with the life-history trait coevolution analysis, we see that increasing gestation lengths are associated with lower degrees of placental invasion and interdigitation, pointing to protection from vertical transmission of pathogens or intergenerational oxidative stress being a likely explanation for some mammalian lineages. An endotheliochorial ancestral state suggests divergence of evolutionary strategies which can be explained by placental protection driving evolution towards lower invasion in some mammalian lineages, whilst the coevolutionary arms race of parent-offspring conflict could direct evolution towards higher placental invasion in others. A labyrinthine ancestral state provides tantalising support for the oxidative shielding hypothesis, yet this will require empirical evidence to substantiate this claim. The placental form is likely governed by many opposing selection pressures far beyond that of increased nutrient provisioning, which may include protection from the negative impacts of exposure to maternal blood, parent-offspring conflict, factors influencing the evolution of body mass and gestation length and parental investment in offspring, which results in the impressive array of placental diversity seen today.

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Author Contributions

EE, JB, MC and AR designed research; EE collected the data; EE, TC and IS analysed data; EE and JB wrote the first draft of the manuscript.

Chapter 3

Untangling the oxidative cost of reproduction: an experimental test in banded mongooses

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Abstract

The cost of reproduction plays a central role in evolutionary theory, but the nature of this cost remains a puzzling question. Oxidative stress has been proposed as a proximate mechanism underlying the cost of reproduction. We examine three pathways by which oxidative stress can shape reproduction. The 'oxidative cost' hypothesis proposes that the reproductive effort generates oxidative stress, while the 'oxidative constraint' and 'oxidative shielding' hypotheses respectively suggest mothers mitigate these costs through reducing reproductive effort or pre-emptively decreasing damage levels. We tested these three hypotheses using data from a long-term provisioning experiment of wild females banded mongooses (*Mungos mungo)*. Our results show that maternal nutritional state did not impact oxidative stress or the production and survival of offspring. We also found that the three oxidative mechanisms likely co-occur during reproduction. We found some evidence of an oxidative cost of reproduction, that mothers attempted to mitigate, potentially by constraining their investment into reproduction, by exhibiting shielding during breeding, or both. This mitigation is likely to be of high importance, as long-term offspring survival was negatively impacted by some markers of maternal oxidative stress. This study shows the importance of longitudinal studies of wild animals to untangle the interconnected mechanisms that shape the cost of reproduction.

Key words: Oxidative stress, *Mungos mungo*, constraint, cost, shielding, reproduction

Introduction

What mechanisms may limit an individual's ability to reproduce? This central question in evolutionary biology has intrigued scientists for decades (Reznick, 1985; Linden and Møller, 1989; Harshman and Zera, 2007). Oxidative stress has been proposed as an important mechanism that might underlie trade-offs in current reproduction, as well as with future reproduction and/or survival (Dowling and Simmons, 2009; Monaghan, Metcalfe and Torres, 2009; Metcalfe and Alonso-Alvarez, 2010). Oxidative stress is a physiological state that arises where reactive oxygen species (ROS), produced as a by-product of metabolism, overwhelm the body's antioxidant machinery that functions to neutralize ROS. Such ROS cause serious damage to biomolecules such as proteins, lipids and DNA, and may ultimately impair cell homeostasis and function (reviewed by Halliwell and Gutteridge, 1999; Speakman *et al.*, 2015). Oxidative stress can thus have highly detrimental consequences on virtually every life-history trait, from growth to ageing (Monaghan, Metcalfe and Torres, 2009; Metcalfe and Alonso-Alvarez, 2010; Speakman *et al.*, 2015).

Researchers have reported a variety of associations between reproduction and oxidative stress. Evidence suggests that individuals with higher levels of oxidative stress prior to reproduction subsequently have lower reproductive output (Costantini, Carello and Fanfani, 2010; Stier *et al.*, 2012; Costantini *et al.*, 2016; Montoya *et al.*, 2016). Thus, oxidative stress might constrain an individual's capacity to invest in reproduction; this has been coined the '*oxidative constraint*' hypothesis.

Many other studies have reported a positive correlation between reproductive effort (in terms of offspring number or size) and oxidative stress (Wiersma *et al.*, 2004; Bertrand *et al.*, 2006; Bergeron *et al.*, 2011; Garratt *et al.*, 2011; Plumel *et al.*, 2014; Dupoué *et al.*, 2020; but see Nussey *et al.*, 2009a; Xu *et al.*, 2014). According to the '*oxidative cost'* hypothesis, this is expected because reproductive investment may result in increased metabolic rate and elevated ROS production (but see Salin *et al.*, 2015).

When considering reproductive state, some studies have found higher levels of oxidative stress in breeders compared to non-breeders (Stier *et al.*, 2017), while several other studies have found the opposite pattern (Garratt *et al.*, 2011; Ołdakowski *et al.*, 2012, 2015; Costantini, Casasole and Eens, 2014; Schmidt, Blount and Bennett, 2014; Vitikainen *et al.*, 2016; Viblanc *et al.*, 2018), or no significant association between reproductive state and oxidative stress (Bertrand *et al.*, 2006). Such inconsistencies led some researchers to highlight potential shortcomings in the design of previous studies (Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014), while others questioned the existence of a proximate link between oxidative stress and reproduction (Speakman and Garratt, 2014; Ołdakowski *et al.*, 2015). Results of recent meta-analyses have suggested that, overall, breeders do indeed exhibit lower levels of oxidative stress compared to non-breeders (Blount *et al.*, 2016). It has been suggested that individuals might pre-emptively decrease oxidative stress levels before they reproduce, in order to shield themselves, and their physiologically-dependent offspring from negative intergenerational consequences of oxidative stress during reproduction (*'oxidative shielding'* hypothesis) (Blount *et al.*, 2016). Indeed, a handful of empirical studies have suggested that maternal oxidative stress during

breeding can negatively impact offspring production and development (Bize *et al.*, 2008; Møller, Karadas and Mousseau, 2008; Essa *et al.*, 2015; Vitikainen *et al.*, 2016; Dupoué *et al.*, 2020). Although mechanisms of shielding are not yet well understood, it is predicted that mothers would incur costs through damage reduction (e.g. by upregulation of antioxidant defences).

Thus, oxidative stress may shape reproduction in various ways: via oxidative costs, oxidative constraints, and oxidative shielding. However, these three mechanisms have rarely been explored in parallel (but see Viblanc *et al.*, 2018), and it remains unclear whether they represent complementary, or alternative paths to optimize lifetime reproductive success. It seems possible that these mechanisms might co-occur. Some level of oxidative cost is likely to be an inevitable consequence of reproduction, which scales with the level of reproductive effort. Oxidative constraint and oxidative shielding, on the other hand, are likely to represent alternative solutions to the same problem – namely the avoidance of excessively high oxidative costs of reproduction that could damage fitness. The former mechanism ensures this by constraining offspring production *per se* and therefore focuses on intra-generational costs of reproduction. The latter mechanism aims to limit deleterious consequences of reproductive investment for maternal and offspring physiological state and therefore raises the additional possibility of inter-generational costs of reproduction. If so, an interesting question is why, and in what circumstances, a mother adopts one strategy instead of another. The nutritional condition of mothers could be a deciding factor. Antioxidants are diverse and include both diet-derived compounds such as vitamins and carotenoids and endogenously

produced molecules such as the enzyme superoxide dismutase (SOD) and the peptide glutathione (GSH) (Halliwell and Gutteridge, 2007). Improved nutrition may therefore allow mothers to allocate more resources towards antioxidant defences (Fletcher *et al.*, 2013; Giordano *et al.*, 2015), and thus potentially to increase offspring production while also ensuring that oxidative stress does not exceed a threshold that would damage fitness. Therefore, in order to understand how oxidative stress shapes reproduction, it is necessary to follow individuals throughout breeding and to examine associations between maternal oxidative stress markers, investment in offspring production for each litter, and the development and survival of offspring. In addition, it is important to investigate how changes in maternal nutrition may alter oxidative state and patterns of reproductive investment.

In order to gain a better understanding of the interplay between maternal nutrition, oxidative stress and reproduction, we conducted a long-term provisioning experiment using wild, female banded mongooses. Banded mongooses live in mixed-sex social groups comprising 5-25 adults (Cant, Vitikainen and Nichols, 2013; Cant *et al.*, 2016). Each social group breeds on average four times a year (Cant, 2000). Each pregnancy lasts approximately 60 days. Breeding females in the same social group give birth synchronously, and all adults communally raise the offspring (Cant, 2000). After birth, pups stay underground in the den for about one month before they emerge. This breeding system allows for powerful split-plot experiment designs, where comparisons can be made between experimental and control individuals while breeding synchronously and in exactly the same environment. For this experiment, two females per social group were provisioned with one egg, three times a week for up to two years, whereas two other age-

matched females were not provisioned and acted as within-group controls. All individuals were weighed and blood sampled regularly. Importantly, foetus size and number were measured using ultrasound scans, and that information was combined to give a pre-natal investment index. Indeed, 50 % of litters fail to emerge from the den for unknown reasons (Cant, 2000), so it is crucial to have a measure of pre-natal investment. Pup body mass was measured at emergence from the den, and offspring survival was monitored for the first 12 months of life.

We predicted that (1) compared to non-provisioned controls, experimental provisioning would allow females to (Figure 1):

(1.1) Allocate more resources towards antioxidant defence, and thus exhibit lower oxidative damage levels during reproduction;

(1.2) Increase offspring production per litter by increasing pre-natal investment and/or offspring survival.

We also (2) aimed to explore how oxidative stress shapes reproduction. We predicted that (Figure 1):

(2.1) *Oxidative cost*: There would be a positive association between levels of reproductive effort (i.e. offspring number and/or size) and subsequent increase in levels of oxidative damage;

(2.2) *Oxidative constraint*: Females with higher levels of oxidative damage before reproduction would invest less in offspring production;

(2.3) *Oxidative shielding*:

(2.3.1) Breeding individuals would exhibit a within-individual decrease in oxidative damage during breeding, leading to lower levels in breeders compared to non-breeders during pregnancy;

(2.3.2) Maternal levels of oxidative stress during pregnancy would be negatively correlated with reproductive investment and/or offspring survival;

(2.3.3) Individuals exhibiting higher levels of oxidative damage before reproduction would exhibit the steepest decrease in oxidative damage during reproduction.

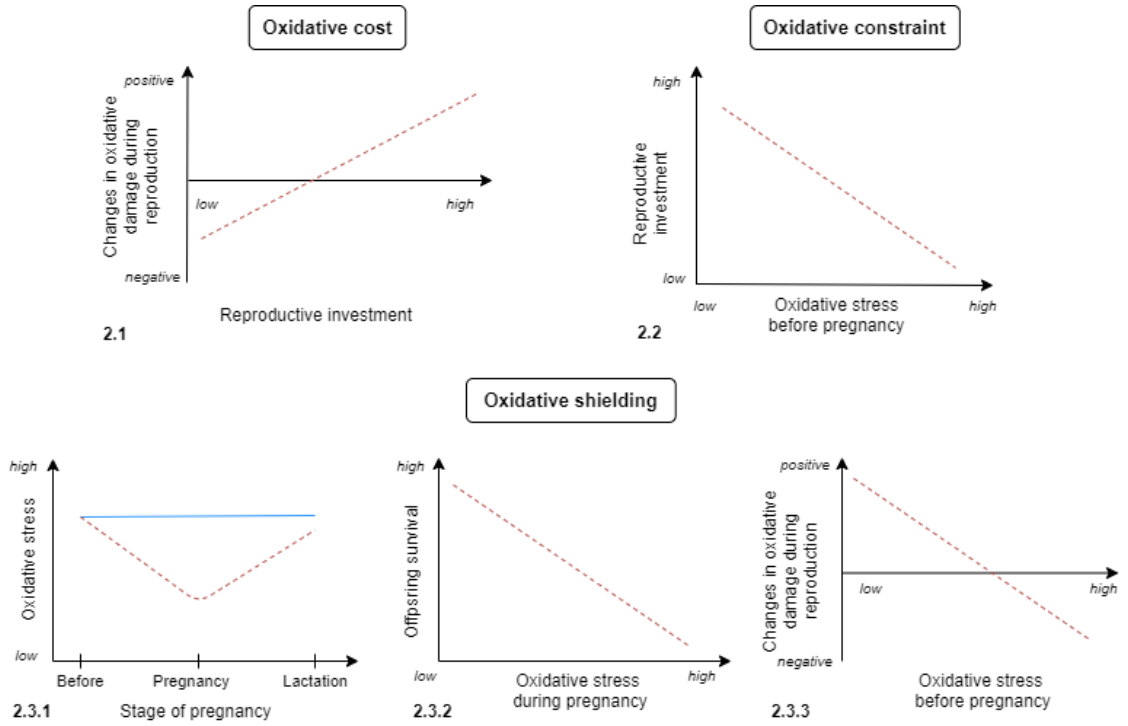

Figure 1: Predictions associated with each hypothesis. 2.1: Oxidative cost, 2.2: Oxidative constraint, 2.3: Oxidative shielding. Red dashed lines represent breeders, blue solid line represents non-breeders.

Methods

Study population

We collected data from a wild population of banded mongooses on the Mweya Peninsula, Queen Elizabeth National Park, Uganda (0°12'S, 29°54'E). Detailed life-history data on this population has been collected continuously since 1995 (Cant, Vitikainen and Nichols, 2013; Cant *et al.*, 2016). Typically, our study population consists of 10-12 social groups that are visited every 1-3 days to record group composition, life history and behavioural data. Banded mongooses always disperse in groups, making it possible to unequivocally distinguish death from dispersal (Cant, Otali and Mwanguhya, 2001). Most individuals are trained to step onto a portable electronic balance in return for a small milk reward and are weighed weekly in the field before morning foraging. Groups containing pregnant females are visited daily to obtain accurate birth dates. Gestation lasts on average 60 days but can range from 57-65 days (Cant, 2000). Individuals in the population are identified using unique shave markings on their back, and PIT tags (TAG-P-122IJ, Wyre Micro Design Ltd., UK) inserted under the skin on the scruff of the neck. Pups are trapped within two weeks of emergence from the den (between 30 and 50 days of age) and anaesthetized using isoflurane. They are then weighed, measured and marked using commercially available blonde hair dye (L'Oreal, UK). A ~2 mm skin sample is collected from the tail tip for genetic assignment of maternity (Sanderson *et al.*, 2015). Individuals within the population are trapped every 3–6 months, using box traps (67 × 23 × 23 cm; Tomahawk Live Trap Co., Tomahawk, WI, USA) and anaesthetized using isoflurane prior to measurements of morphometrics, ultrasound scans and collection of blood samples.

Experimental provisioning of females

Experimental provisioning was conducted in 6 groups between May 2017 and March 2020. Provisioned females were fed with one egg, gently cooked as an omelette, three times a week. Each provisioned female was associated with an aged-matched within-group control female that remained non-provisioned. If an experimental female died, another female from the group was selected as a replacement. A total of 18 females were provisioned, and 15 females acted as controls, for a mean duration of 561 days (min=165 days; max= 1053 days). 71 communal litters were born during the experimental period, with an average of 6.7 individual litters contributed to communal litters by each female (min=2; max=15).

Ultrasound Scanning

We carried out ultrasound scans of foetuses carried by pregnant females to measure pre-natal offspring production. The ultra-sound scans were taken around day 25 of pregnancy (mean +/- SE = 25.29+/- 0.67 days). A Sonoscape S6BW ultrasound scanner with an L742 linear probe (Vet Image solutions, UK) was used to obtain cross-sectional images of each foetus along their transverse plane in their gestational sac at their widest point. Scans were not used if the image was unclear, if the foetus was cut off the edge of the image or if the gestational sacs were not elliptical in shape. Perpendicular measurements of the gestational sac were taken using ImageJ (Schneider, Rasband and Eliceiri, 2012), where measurement ‘B’ was taken along the longest axis of the gestational sac at 90° from measurement ‘A’ (see example in Supplementary Figure 1). The

cross-sectional area was then calculated following the methods of (Inzani *et al.*, 2016) using the formula: cross-sectional area = (A/2) x (B/2) x π.

Pre-natal investment

An index of pre-natal investment was computed by calculating the mean foetus size measured *in utero* for each female for a given pregnancy using ultrasound scans, multiplied by the number of foetuses carried by each female. To account for differences in the exact day the scan was taken compared to the date of birth, foetus size was divided by the age at measure relative to the date of birth. We used the mean foetus size for a given pregnancy for each female, as it was sometimes not possible to measure accurately the size of each foetus.

Collection of blood samples

Blood (volume 100-500 µl) was collected from the jugular vein using a 25G needle and syringe and transferred to a 3 ml EDTA BD Vacutainer®. Whole blood was centrifuged at 2000 x g for 4 min at 4°C (Spectrafuge mini centrifuge, Sigma Aldrich, UK) to separate the plasma, which was frozen for analyses of malondialdehyde (MDA) and protein carbonyls (PC). Samples of red blood cells (RBC) were frozen for analysis of glutathione (GSH) and superoxide dismutase (SOD). All samples were snap-frozen in liquid nitrogen within 10 minutes of collection, and subsequently transported to our UK laboratory in a cryogenic shipper (Taylor-Wharton CX100, Jencons, UK) and stored at -80°C until analysis. Sampling occurred *before pregnancy*: between 80 days and 50 days before the litter's birth date [mean +/- SE = -60.5+/- 0.8 days]; *during pregnancy*: between 49 days and 0 days before the litter's birth date [mean +/- SE = -24.3+/- 1.13

days]; or *during lactation*: between the litter's birth date and 30 days after [mean +/- SE = 14+/- 0.8 days].

Quantification of oxidative stress markers

Lab analyses were performed blindly with respect to sample identity and the experimental design. All steps were conducted on ice to minimise oxidation. All chemicals were HPLC (high-performance liquid chromatography) grade, and chemical solutions were prepared using ultra-pure water H_2O MQ (Milli-Q Synthesis; Millipore, Watford, UK). Assays were conducted within one year of collection (time since collection (mean +/- SE): MDA : 268 +/- 89 days; PC : 295+/- 94 days; SOD : 286 +/- 58 days; GSH : 295 +/- 60 days).

Malondialdehyde (MDA)

Plasma malondialdehyde was determined using an HPLC with a fluorescence detector (Agilent 1000; Agilent Technologies, USA). We followed the method in (Nussey *et al.*, 2009) with some modifications. Details about the method can be found in the Supplementary Material. MDA level in sample is expressed in µM; the coefficient of variation computed for 88 duplicate samples was 9.5%.

Protein Carbonyls (PC)

Plasma protein carbonyls, a marker of protein oxidative damage, were measured using a colorimetric assay following a protocol adapted from the Carbonyl Assay Kit (Cayman Chemical Company), using a plate reader (Spectramax M2;

Molecular Devices, USA). Due to limitations in the amount of plasma available as well as the high protein content of samples, 50 µl of plasma was used in the sample and control tubes instead of the 200 µl recommended in the kit instructions. Carbonyl content in samples was expressed in nmol/mg protein in the controls; the coefficient of repeatability for duplicate samples was 12.5%, computed on 65 samples.

Superoxide Dismutase (SOD)

We assessed SOD activity (U/ml) in RBC samples using the Cayman chemicals superoxide dismutase assay kit (Cayman Chemical Company, USA). RBC samples were diluted in a 1:10 w/v solution using ice-cold H_2O MQ, then centrifuged at 10 000 x g for 15 min at 4°C. The supernatant was collected and further diluted 1:100 with sample buffer for quantification. The quantification is based on the detection of superoxide radicals generated by xanthine oxidase and neutralized by SOD, using a plate reader (Spectramax M2; Molecular Devices, USA). One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The coefficient of variation computed for 40 duplicate samples was 10.8%.

Glutathione (GSH)

We assessed reduced glutathione levels in RBC samples using the Cayman chemicals glutathione assay kit (Cayman Chemical Company, USA). Similar to the SOD assay, RBC samples were diluted in a 1:10 w/v solution using ice-cold H_2O MQ, then centrifuged at 10 000 x g for 15 min at 4°C. The supernatant was collected and deproteinated before the assay. The quantification is based on the

detection of 5-thio-2-nitrobenzoic acid (TNB), produced by a reaction between the sulfhydryl group of GSH and 5,5'-dithio-bis-2-nitrobenzoic acid (DNTB), using a plate reader (Spectramax M2; Molecular Devices, USA). Reduced glutathione level is expressed in µM; the coefficient of variation computed for 38 duplicate samples was 11.9%.

Statistical analyses

Data were analysed using R, version 3.6.1 (R Core Team, 2016). The 'lme4' package was used for linear mixed-effects models (Bates *et al.*, 2019), while the 'lmerTest' package was used to obtain p-values (Kuznetsova, Brockhoff and Christensen, 2017). The 'stats' package was used for generalized linear models (R Core Team, 2016), while the 'coxme' package was used to run survival mixed-effects cox models (Therneau, 2020). The 'emmeans' package was used to perform posthoc tests, with false discovery rate correction for test multiplicity (Lenth *et al.*, 2020). For all analyses, we checked model assumptions, *i.e.* normality, linearity, homoscedasticity and proportional hazards for survival analysis. The significance level was set at 0.05, and the false discovery rate procedure was applied to account for the multiplicity of tests (Benjamini and Hochberg, 1995). When required, litter identity was included as a random factor, to control for any litter effects such as environmental variation or differences between packs. Maternal identity was included to avoid pseudo-replication, as mothers may have had several pups within the same litter or may have participated in several litters. Random intercept models were used, and random effects are additive. Initially, social group identity was also included as a random effect in these models but was found to have no influence on the results. As such, we decided to remove this from the model to reduce the number of variables.

An interplay is likely to occur between maternal nutrition, oxidative stress and reproduction. However, testing for an interaction between maternal provisioning treatment and reproductive investment/offspring survival or oxidative stress markers would be statistically dubious. Indeed, we predicted that the provisioning treatment would impact both oxidative stress markers and maternal investment/offspring survival. Therefore, including both the provisioning treatment and oxidative stress markers or the provisioning treatment and reproductive investment/offspring survival as explanatory variables in the same model would result in a high risk of multicollinearity, as checked using the variance inflation factor (VIF), which represents a major issue for the interpretation of linear models (Kraha *et al.*, 2012). Therefore, we first examined the effect of the treatment on maternal oxidative stress markers and reproductive investment/offspring survival. Then, we examined oxidative stress data to understand the impact on maternal investment/offspring survival, and thus evaluate whether oxidative stress shaped reproduction.

1. Consequences of maternal provisioning experiment

1.1 Oxidative stress markers during breeding

We tested the effect of maternal provisioning treatment on oxidative stress marker dynamics during the breeding event in pregnant females. To do so, we ran linear mixed effect models with oxidative stress markers (either PC, MDA, SOD or GSH) as a response variable, with the timing of measurement (either *before pregnancy*, *during pregnancy* or *during lactation*), maternal provisioning treatment, and their interaction as explanatory variables. Litter identity and

maternal identity were included as random effects. Day of sampling (relative to the date of birth) was initially included as a covariate. However, it was never significant, and as such was removed from the final models to keep them as simple as possible.

We did not use data reduction methods such as PCA with oxidative markers for two reasons. First, we did not have oxidative stress measures for all markers for each individual because of blood sample volume limitations. Second, the markers of oxidative stress were only very weakly correlated. Indeed MDA showed a Pearson's correlation coefficient of r=-0.004 (P-value=0.95) with PC, r=-0.08 (P-value=0.16) with SOD and r=-0.08 (P-value=0.15) with GSH. SOD showed a correlation coefficient of r=-0.06 (P-value=0.31) with PC and r=-0.10 (P-value=0.08) with GSH, while PC and GSH had a correlation of r=0.06 (P-value=0.28). Given such weak correlations, PCA did not provide easily interpretable principal components. Therefore, we decided to use individual markers in our models.

1.2 Maternal investment and offspring survival

To examine the effect of the maternal provisioning treatment on maternal investment and offspring survival, we ran linear mixed effect models with either pre-natal investment, offspring body mass at emergence from the den or number of offspring emerging from the den as a response variable, and maternal provisioning treatment as an explanatory variable. For the model with offspring body mass at emergence as a response variable, to account for differences in the age at which body mass was measured, age at measurement was included

as a covariate. For the model with the number of offspring emerging from the den as a response variable, the number of foetuses carried per female was included as a covariate. Litter identity and maternal identity were included as random effects. For the results of an analysis to determine whether the maternal provisioning treatment had any influence on foetus size alone, see table S7 of Appendix B.

The impact of the maternal provisioning treatment on survival to 12 months was determined using a Cox proportional hazard model, with survival to 12 months as a response variable, and the maternal provisioning treatment as the explanatory variable. Litter identity and maternal identity were included as random effects.

2. How does oxidative stress shape reproduction?

2.1 Test of the oxidative cost hypothesis

To check whether reproduction represents an oxidative cost, we ran linear mixed models with within-individual changes in levels of oxidative stress markers (PC, MDA, SOD or GSH) during the breeding event as a response variable. Within-individual changes were calculated as the difference between the marker levels during pregnancy and the levels before pregnancy. These differences were adjusted to account for potential regression towards the mean, a phenomenon where extreme values in a first measure are likely to be closer to the mean in a second measure. They were adjusted following Kelly and Price (2005). Pre-natal investment and offspring body mass at emergence from the den were used as explanatory variables. To account for differences in the age at which body mass

was measured, body mass was divided by the age at measurement. Litter identity and maternal identity were included as random effects.

2.2 Test of the oxidative constraint hypothesis

To explore whether maternal investment in reproduction is constrained by maternal oxidative stress levels prior to reproduction, we ran two linear mixed models, with either pre-natal investment or offspring body mass at emergence from the den as response variables and all oxidative stress markers (PC, MDA, SOD and GSH) measured before pregnancy as explanatory variables. For the model with offspring body mass at emergence as a response variable, to account for differences in the age at which body mass was measured, age at measurement was included as a covariate. Litter identity and maternal identity were included as random effects.

2.3 Test of the oxidative shielding hypothesis:

2.3.1. Changes in oxidative stress markers over the course of reproduction in breeders compared to non-breeders

To understand the effect of breeding status (breeders versus non-breeders) on oxidative stress markers dynamics during the breeding event, we ran linear mixed effect models with oxidative stress markers as a response variable (either PC, MDA, SOD or GSH) with the time of measurement (either *before pregnancy*, *during pregnancy* or *during lactation*), breeding status, and their interaction as explanatory variables. For non-breeders, time of measurement was assigned based on the date of the breeding event for the social group they belong to. Day of sampling (relative to the date of birth) was initially included as a covariate.

However, it was never significant, and as such was removed from the final models to keep them as simple as possible. Litter identity and maternal identity were included as random effects.

2.3.2. Does oxidative stress level during reproduction impact maternal investment and offspring survival?

To check whether maternal oxidative stress levels were related to offspring fitness, we ran several linear mixed models. Pre-natal investment, offspring body mass at emergence from the den and number of offspring emerging from the den were used as a response variable, and all oxidative stress markers (PC, MDA, SOD and GSH) measured during pregnancy as explanatory variables. For the model with offspring body mass at emergence as a response variable, age at measurement was included as a covariate to account for differences in the age at which body mass was measured. Litter identity and maternal identity were included as random effects.

The impact of maternal oxidative stress levels during pregnancy on survival to 12 months was investigated using a Cox proportional hazard model, with survival to 12 months as a response variable, and all oxidative stress markers (PC, MDA, SOD and GSH) measured during pregnancy as explanatory variables. Litter identity and maternal identity were included as random effects.

2.3.3. Are within-individual changes in oxidative stress markers correlated with baseline levels prior to reproduction?

To explore whether individuals adjusted their oxidative stress levels during the breeding event based on their baseline levels, we ran linear mixed models with within-individual changes in levels of oxidative stress markers (PC, MDA, SOD or GSH) during the breeding event as a response variable, calculated as the difference between marker levels during pregnancy and levels before pregnancy. These differences were adjusted to account for regression towards the mean according to Kelly and Price (2005). We used levels of oxidative stress markers before reproduction as explanatory variables, with body mass before pregnancy as a covariate, in an attempt to assess whether female condition might influence that relationship. Litter identity and maternal identity were included as random effects.

Results

1. Consequences of maternal provisioning experiment

1.1 Oxidative stress markers during breeding

In breeders, levels of protein carbonyls varied according to the stage of reproduction (Table S1), with posthoc tests showing lower levels during pregnancy compared to lactation (T-ratio=-2.7, P-value=0.02). However, levels of protein carbonyls did not differ significantly between provisioned and non-provisioned females, or according to the interaction between stage of reproduction and maternal provisioning treatment (Table S1, Figure 2). Levels of MDA, SOD and glutathione did not differ significantly in relation to the stage of reproduction, maternal provisioning treatment or their interaction (Table S1, Figure 2).

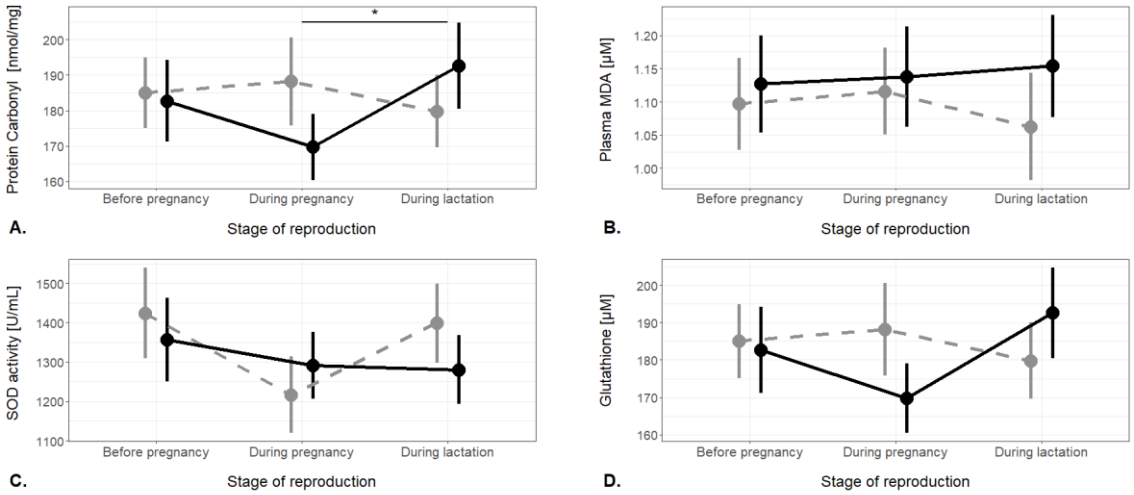

Figure 2: Dynamics of oxidative stress markers during the breeding event for pregnant females. A. Protein Carbonyl level, B. MDA level, C. SOD activity, D. Glutathione level. Black dots and solid lines represent provisioned females, while grey dots and dashed lines represent non-provisioned females. Symbols represent raw data means +/- SE. Stars indicate statistical significance.

1.2 Maternal investment and offspring survival

Maternal provisioning treatment did not significantly affect reproductive investment in terms of pre-natal investment, nor offspring body mass at emergence while controlling for the age at measurement (Table S2). Moreover, we did not find a significant effect of maternal provisioning treatment on offspring survival. The number of offspring emerging from the den was predicted by foetus number similarly in both provisioning treatments, while the effect of maternal provisioning treatment on offspring survival to 12 months was not statistically significant (Table S2, Figure 3).

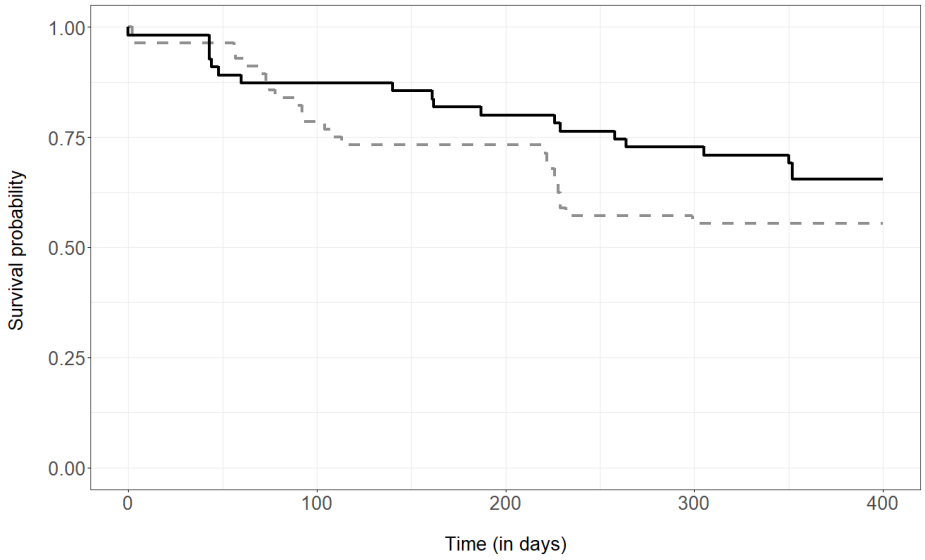

Figure 3: Survival of offspring from provisioned and non-provisioned mothers. The black line represents the offspring of provisioned mothers, while the grey dashed line represents the offspring of non-provisioned mothers.

2. How does oxidative stress shape reproduction?

2.1 Test of the oxidative cost hypothesis

Females that invested more in foetus production showed an increase in glutathione levels during the breeding event (Table S3, Figure 4). However, intra-individual changes in levels of other markers of oxidative stress were not

significantly predicted by pre-natal investment or offspring body mass at emergence (Table S3).

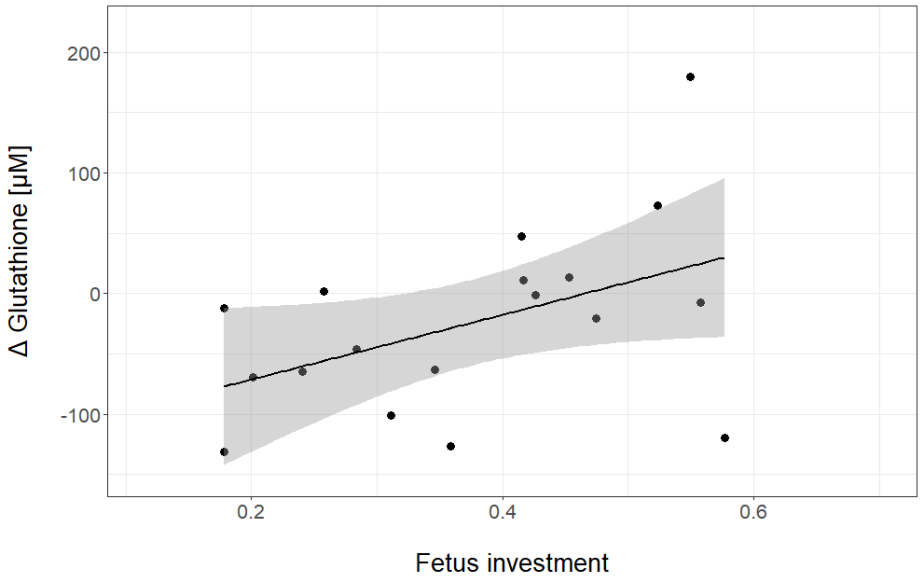

Figure 4: Relationship between within-individual changes in glutathione levels and pre-natal investment. The line represents the regression line +/- 95% confidence interval (shaded region) with points representing the raw data.

2.2 Test of the oxidative constraint hypothesis

Maternal glutathione level before pregnancy positively predicted pre-natal investment (Table S4, Figure 5). However, pre-natal investment was not significantly predicted by any other oxidative stress marker, although pre-natal investment showed a marginally non-significant positive relationship with PC levels (Table S4).

Moreover, maternal SOD activity before pregnancy positively predicted offspring body mass at emergence (Table S4, Figure 5), although that relationship did not remain significant when data points with SOD activity greater than 2500 were removed. Offspring body mass at emergence was not significantly predicted by any other markers of oxidative stress (Table S4).

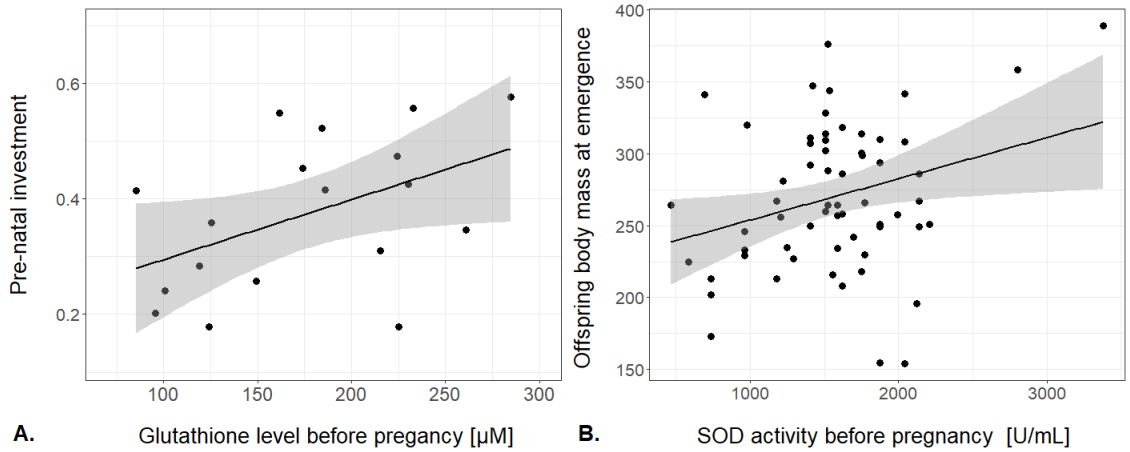

Figure 5: Relationships between maternal reproductive investment and oxidative stress markers before pregnancy. A. Pre-natal investment in relation to glutathione levels before pregnancy; B. Offspring body mass at emergence from the den in relation to SOD activity before pregnancy. Removal of the data points with SOD activity greater than 2500 renders the relationship no longer significant. The line represents the regression line +/- 95% confidence interval (shaded region) with points representing the raw data.

2.3 Test of the oxidative shielding hypothesis:

2.3.1. Changes in oxidative stress markers over the course of reproduction in breeders compared to non-breeders

Protein carbonyl levels varied in relation to the interaction between breeding status and stage of reproduction. Post-hoc tests showed that protein carbonyl levels were similar in breeders and non-breeders before the breeding event, but differed during pregnancy, with breeders exhibiting significantly lower levels of protein carbonyl compared to non-breeders (T-ratio = 3.42, P-value< 0.001). Breeders showed an increase in protein carbonyl levels during lactation compared to levels during pregnancy (T-ratio= -2.28, P-value=0.06) (Table S5, Figure 6).

MDA, SOD and glutathione did not differ significantly in relation to the stage of reproduction, the breeding status or their interaction (Table S5, Figure 6).

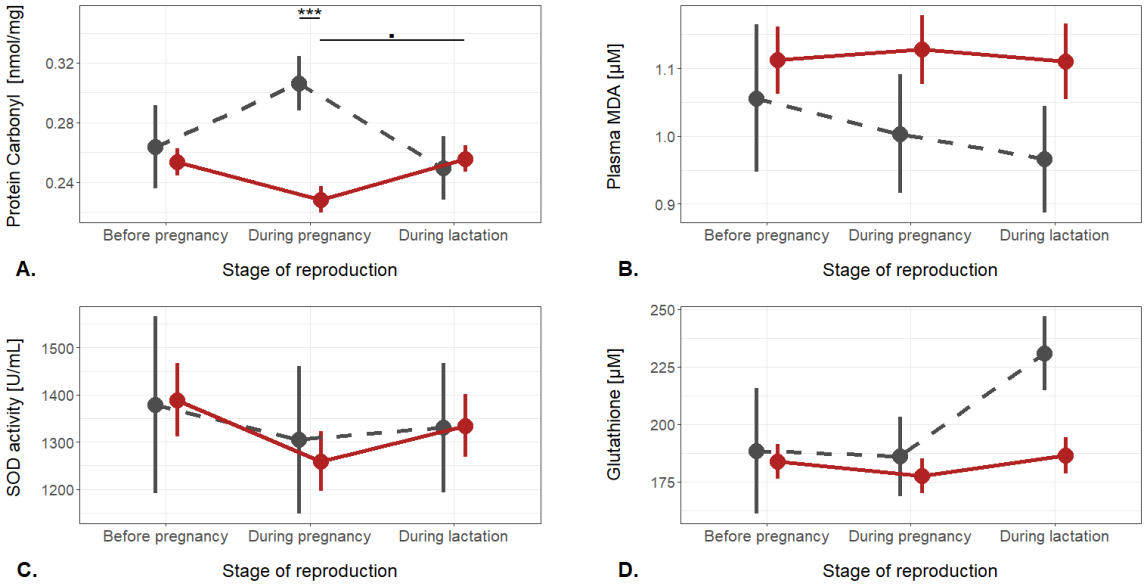

Figure 6: Dynamics of oxidative stress markers during the breeding event. A. Protein Carbonyl level, B. MDA level, C. SOD activity, D. Glutathione level. Red dots and solid lines represent breeding females, while grey dots and dashed lines represent non-breeding females. Symbols represent raw data means +/- SE Stars indicate statistical significance: ***: P-value<0.001, •: P-value= 0.06.

2.3.2. Do oxidative stress levels during pregnancy influence maternal investment and offspring survival?

Pre-natal investment, offspring' body mass at emergence and survival to emergence were not significantly impacted by maternal levels of oxidative stress during pregnancy (Table S6). Interestingly, survival to 12 months was negatively correlated with maternal levels of protein carbonyls during pregnancy, while positively correlated to both glutathione and MDA (Table S6, Figure 7).

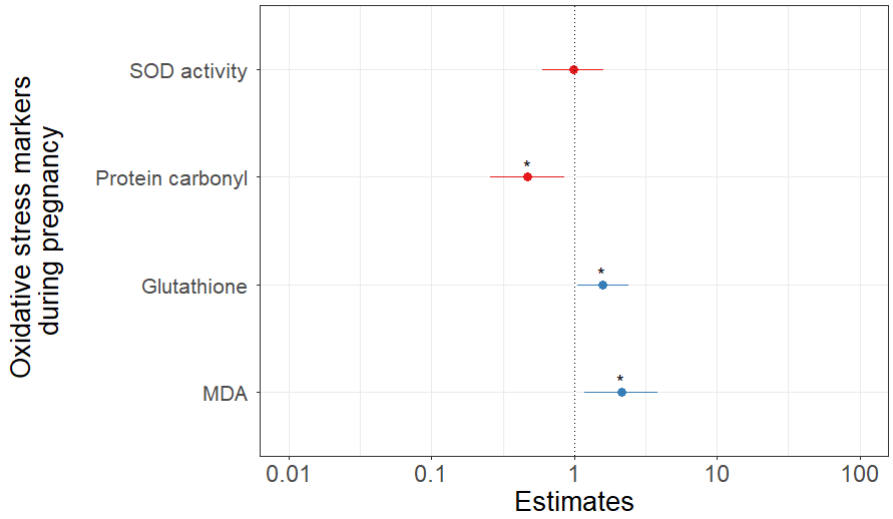

Figure 7: Correlation between survival to 12 months and maternal oxidative stress markers measured during pregnancy. Hazard ratios are shown. * Indicates statistical significance.

2.3.3. Are within-individual changes in oxidative stress markers correlated with baseline levels prior to reproduction?

After adjusting the values of the within-individual changes in oxidative stress markers for the regression towards the mean (Kelly and Price 2005), we found that these intra-individual changes were not predicted by baseline levels prior to reproduction (PC: estimate +/- SE = -0.16 +/- 0.13, F-value $_{1,56.2}$= 1.38, P-value= 0.24; MDA: estimate +/- SE = 0.11 +/- 0.11, F-value $_{1,64.8}$= 1.02, P-value= 0.31; SOD: Estimate +/- SE = -0.11 +/- 0.11, F-value $_{1,49.6}$ = 0.97, P-value= 0.33; GSH: estimate +/- SE = 0.003 +/- 0.12, F-value $_{1,60.46}$<0.01, P-value= 0.97).

Discussion

We explored how oxidative stress can shape reproduction, by testing three mechanisms: oxidative cost, oxidative constraint and oxidative shielding. Using a long-term provisioning experiment, we also tested the linkages between maternal nutrition, oxidative stress and offspring production.

We predicted that experimental provisioning would improve individuals' oxidative state, either by allowing increased endogenous antioxidant production and/or by providing exogenous antioxidants, as eggs are rich in antioxidants including vitamin E (Seuss-Baum, 2007; Nimalaratne and Wu, 2015). However, in contrast with previous studies (Fletcher *et al.*, 2013; Giordano *et al.*, 2015), we found no significant effect of the dietary provisioning of females on their oxidative state. Moreover, provisioning did not affect the offspring production by females. What then did provisioned mothers do with the extra resources that they received? It is unlikely that our experimental provisioning represented a negligible input to the diet, as one egg represents about one-third of daily energy requirements (Laver *et al.*, 2020). It is possible that fed individuals subsequently reduced their foraging effort, thus leading to similar nutrition in provisioned and non-provisioned individuals. A more likely potential explanation is that natural food availability could have been relatively high during the experiment, thus obscuring the effect of provisioning, as shown in a meta-analysis based on studies of birds (Ruffino *et al.*, 2014). Indeed, the mongoose population density was extremely low at the beginning of the experimental period, and increased slowly, which likely conferred unusually profitable foraging opportunities. In general, the effect of provisioning on wild animals is poorly understood. While food supplementation in

wild animals often leads to a decrease in home range size, an increase in body mass, and advances the date of first breeding, it has limited impact on offspring production (Boutin, 1990). Similar results were found in a study of the effects of feeding on anthropogenic food waste by banded mongooses; individuals that fed on refuse were heavier and carried more foetuses (Otali and Gilchrist, 2004). However, despite these apparently beneficial effects, conception rate, number of emerging offspring and survival to 3 months of age were similar in refuse-feeding and non-refuse feeding groups, suggesting that extra nutritional resources do not necessarily lead to increased reproductive success in banded mongooses (Otali and Gilchrist, 2004).

Our results provide some evidence that oxidative stress can shape reproduction in multiple ways. First, we found limited evidence for the 'oxidative cost' hypothesis, as increased investment in reproduction was associated with increased levels of the antioxidant glutathione. Such increase in antioxidant defences associated with stable levels of oxidative damage suggests an upregulation of antioxidant defences in response to an oxidative challenge, to prevent an increase in oxidative damage (Costantini and Verhulst, 2009; Hõrak and Cohen, 2010; Beaulieu and Costantini, 2014). A similar pattern was reported in zebra finches (*Taeniopygia guttata*) after an oxidative challenge induced by diquat dibromide, which elicited an increase in antioxidant capacity, while it did not affect damage levels (Tomášek *et al.*, 2016). Thus, our results suggest that increased offspring production is likely to pose an oxidative challenge. Since these are correlative data it is perhaps not surprising that we found only limited evidence for an oxidative cost of reproduction, as individuals are likely to adjust offspring production to their own condition. Second, we found some support for

the ‘oxidative constraint’ hypothesis. Females with higher levels of antioxidant defences prior to breeding subsequently invested more heavily in reproduction. This result adds to pre-existing empirical evidence supporting the oxidative constraint hypothesis (Stier *et al.*, 2012; Costantini *et al.*, 2016; Montoya *et al.*, 2016).

Finally, we found some potential support for the ‘oxidative shielding’ hypothesis, with plasma levels of protein carbonyls, a marker of damage to proteins, being lower in pregnant females compared to non-breeders. Maternal levels of protein carbonyls during pregnancy were also negatively correlated with offspring survival to one year of age. This suggests that protein carbonyls may have detrimental consequences which could be transmitted across generations, hence the importance of maintaining low levels of protein carbonyls during pregnancy. A similar negative effect of protein carbonyls on survival has been reported in Soay sheep (*Ovis aries*), where male lambs with higher plasma levels of protein carbonyls had lower survival to the first winter (Christensen *et al.*, 2015). Moreover, we found that maternal levels of the antioxidant glutathione correlated positively with offspring survival to one year of age, again suggesting long-term intergenerational consequences of maternal oxidative state. Surprisingly, levels of oxidative lipid damage (MDA) in pregnant females were also positively correlated with offspring survival to one year of age. However, levels of MDA, glutathione and protein carbonyls were not significantly correlated. This pattern is opposite to what was previously found in banded mongooses, where maternal MDA levels correlated negatively with mixed-maternity litter survival (Vitikainen *et al.*, 2016). However, in Vitikainen et al.’s (2016) study, levels of MDA during pregnancy were markedly and significantly higher than the present study

(Vitikainen *et al.*, 2016: mean +/- SE= 1.74 +/- 0.05; this study: mean +/- SE= 1.11 +/- 0.03; T-value=9.16, P-value<0.001). Negative impacts of oxidative stress may not arise at relatively low levels of lipid peroxidation. Alternatively, a hormesis effect could be suggested, where early exposure to mild levels of oxidative stress could have a long-term beneficial effect (Costantini, 2014; Alonso-Alvarez, Canelo and Romero-Haro, 2017; Losdat *et al.*, 2018). Unfortunately, these correlative findings do not necessarily indicate causation due to the failure of the experimental provisioning to alter oxidative state.

Interestingly, our data propose that oxidative costs and constraints may be observed in terms of variation in antioxidant levels rather than oxidative damage, suggesting that individuals were mostly able to mitigate against the increased risk of oxidative stress in association with reproduction. Additionally, we report for the first time a decrease in protein carbonyl levels during pregnancy. Indeed, earlier work has reported elevated protein carbonyl levels during breeding in banded mongooses (Vitikainen *et al.*, 2016), and Brandt's voles *(Lasiopodomys brandtii)* (Xu *et al.*, 2014), and as a result of meta-analysis (Blount *et al.*, 2016). This suggests that maintaining low levels of protein carbonyls during reproduction might rarely be possible, perhaps only where individuals are in good condition and experiencing relatively low levels of oxidative stress. Together with our finding that MDA levels were low and positively correlated with offspring survival, these results suggest that environmental conditions were rather benign during the experiment, thus allowing individuals to maintain low levels of oxidative stress, even during reproduction.

The change in circulating levels of oxidative stress markers during breeding once corrected for regression towards the mean, appeared to be independent of the levels measured prior to reproduction. Such absence of linkage suggests that, contrary to our predictions, individuals do not adjust levels of oxidative stress markers during breeding in relation to their baseline levels. Potentially, such mitigation might be too costly for individuals of lower quality, which might be expected to display relatively high baseline levels of oxidative stress.

One caveat of this study is that these findings ought to be interpreted with caution. When correcting for false discovery rate, we find that all significant values become non-significant. In this case, we cannot categorically reject the null hypothesis and therefore cannot draw robust conclusions about each hypothesis tested here. When conducting studies where many repeated tests are performed involving a large number of variables, controlling for false discovery rate provides a compromise between type I and type II error (García, 2003; Nakagawa, 2004). However, what represents a 'large number' is currently debated, even amongst statisticians. With a maximum of four tests per hypothesis (one for each marker), our results do not necessarily require this type of correction, although we have chosen to include it for the information of the reader. Taking this into consideration, at a minimum, our findings highlight observable trends between markers of oxidative damage/antioxidants and maternal investment and offspring survival. However, we believe that this study still provides an important contribution to the disentangling of hypotheses attempting to explain the cost of reproduction, although we leave the absolute interpretation of this to the reader's discretion.

Longitudinal sampling across the annual calendar and including breeding events can give powerful insights into how oxidative stress may shape reproduction. Indeed, it is now well established that breeding individuals often vary considerably in baseline levels of oxidative stress (Herborn *et al.*, 2011; Alajbeg *et al.*, 2017; Martinez-Moral and Kannan, 2019; Bodey *et al.*, 2020). Within-individual changes in oxidative stress levels associated with reproductive effort are therefore more informative than stand-alone measurements during a reproductive episode. Longitudinal data are also essential for testing the oxidative shielding hypothesis, which predicts decreased oxidative damage levels during stages of reproduction when offspring are physiologically-dependent on their mothers. The present study has illustrated the value of combining different sampling time points, including single time point measurements and intra-individual changes in order to investigate how oxidative stress may shape reproduction (see also Viblanc *et al.*, 2018). More specifically, oxidative stress levels before reproduction can allow tests of the oxidative constraint hypothesis, while oxidative stress levels during breeding can inform about the oxidative cost hypothesis and the oxidative shielding hypothesis.

In conclusion, our results provide limited evidence for the view that oxidative stress is an important factor that shapes reproduction. Oxidative stress is a proximate cost of reproduction and may have negative intergenerational consequences. As such, individuals should avoid high levels of oxidative damage when breeding, we propose that this could be done either by tailoring their investment in reproduction to their baseline oxidative state and/or by lowering levels of damage and increasing antioxidant protection during reproduction. Indeed, oxidative constraint and oxidative shielding are perhaps unlikely to be

alternative mechanisms by which oxidative stress can shape reproduction. Rather, these mechanisms possibly co-occur in order to optimize an individual's lifetime reproductive success.

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Author Contributions

MM, FT, HM, RJ, MC and JB planned and supervised data collection; MM, LH, ED, CM performed sample analysis; EE measured the foetus size and wrote ultrasound protocol; HN and GL constructed the pedigree; MM and JB analysed the data and wrote the manuscript; All authors gave final approval for publication.

Chapter 4

General discussion

In this thesis, I sought to advance our knowledge of the negative consequences of intergenerational effects in mammals. To do this, I started by examining an organ responsible for their transmission – the placenta. By reconstructing the ancestral state of placentation in mammals, I explored the associations between placental traits and life-history traits with evolutionary context. This enabled me to investigate whether protection from intergenerational effects could be a possible driver for placental evolution. Complimentary to this, we experimentally tested different hypotheses surrounding the oxidative cost of reproduction and the negative intergenerational impact this could have by experimentally manipulating nutrition in a population of wild banded mongooses. This allowed us to further investigate a possible mechanism behind the trade-off between reproduction and survival (the cost of reproduction), and examine the potential consequences of this mechanism for the future generation.

Mammalian placentation

The comparative analysis of mammalian placentation explored associations between life-history strategy and placental traits. I tested three main hypotheses regarding the evolution of the placenta; the 'cooperation/altruism' hypothesis whereby the placenta evolved to facilitate efficient nutrient transfer to the offspring *in utero*, the 'parent-offspring conflict' hypothesis where placental evolution is driven by the antagonistic coevolution of mother and foetus over control of maternal resources, and the 'protection' hypothesis where the evolution of lower invasion and interdigitation could provide a protective role, either from the vertical transmission of pathogens or oxidative damage. Invasion and interdigitation were the key placental traits studied, in conjunction with the following life-history traits:

maximum longevity, gestation length, litter size, body mass and mating system. To provide evolutionary context to this study, trait switching analyses were also performed on the placental types to establish the order of evolution and infer ancestral state.

This study of placental and life-history traits revealed that placentation is not directly associated with the pace of life, contrary to other previous findings (Pires, McBride and Reznick, 2007; Lewitus and Soligo, 2011; Pires *et al.*, 2011; Garratt, Gaillard, *et al.*, 2013). Once phylogenetic relatedness and body mass were controlled for, we only saw an association between gestation length, placental invasion and interdigitation, where shorter gestation lengths were associated with a higher degree of both placental invasion and interdigitation. This agrees with our prediction supporting the 'protection' hypothesis. However, the key placental trait that explained gestation length was placental interdigitation, which agrees with the findings of Capellini, Venditti and Barton (2011), as this is thought to influence the rate of nutrient transfer across the placenta having a knock-on effect on foetal growth rates and thus gestation length (Wildman *et al.*, 2006; Capellini, Venditti and Barton, 2011). As it is hypothesised that intergenerational effects have the ability to influence species' life-history, the overall lack of an association between the pace of life and placentation found by this study disagree with Pires' life-history facilitation hypothesis (Pires *et al.*, 2011). This perhaps suggests that variation in placental invasiveness and interdigitation are not the key placental traits responsible for directly facilitating life-history evolution or *vice versa*, and thus are traits that have a minimal influence on the transmission of negative intergenerational effects. Indeed, vertical damage transmission is not necessarily limited to transmission via the placenta; oxidative stress may also be transferred

from mother to offspring via milk. Lactation is known to place a high nutritional demand on the mother which increases the production of ROS and thus the risk of oxidative stress (Schäff *et al.*, 2012). Not only does this have the potential to reduce the antioxidant content of milk and colostrum at a critical time when the offspring are first exposed to atmospheric oxygen (Castillo *et al.*, 2005; He *et al.*, 2008; Albera and Kankofer, 2009; Rizzo, Ceci, *et al.*, 2013), but may also reduce milk yields (Suriyasathaporn *et al.*, 2009; Chandra *et al.*, 2013). Here there is the potential to indirectly influence oxidative stress levels experienced by neonatal offspring. Additionally, malondialdehyde (MDA, a marker for oxidative damage) can be found in milk (Wicheansoni *et al.*, 2007; Bouwstra *et al.*, 2008; Suriyasathaporn *et al.*, 2009; Rizzo, Pantaleo, *et al.*, 2013), implying another direct pathway for vertical oxidative damage transmission.

However, the striking association between body mass and placental invasion found in this comparative analysis seemingly contradicts the findings above. Smaller body masses are associated with higher placental invasion, even when controlling for phylogenetic relatedness and other life-history traits. This pattern is broadly the same for placental interdigitation except when controlling for gestation length, implying that gestation length explains the association we see between placental interdigitation and body mass. Again, we find support for our 'protection' hypothesis. As body mass is thought to be a key variable that influences life-history traits, this association between body mass and placentation suggests a concomitant association with life-history traits (Western, 1979; Peters, 1983; Schmidt-Nielsen, 1983; Dobson and Oli, 2007). Perhaps the plastic nature of life-history traits can explain why we do not observe a direct association between placentation and life-history strategy (Stearns, 1992; Roff, 1997;

Pigliucci, 2001; Dewitt and Scheiner, 2004). Smaller mammals, which often exhibit a fast pace of life, have tissues that are far more metabolically active when considering weight-specific metabolic rate (Western, 1979; Schmidt-Nielsen, 1997; Hill, Wyse and Anderson, 2012). As oxygen transport between mother and foetus is thought to be limited by the placenta (Rahn, 1982; Paganelli and Rahn, 1984; Singer and Mühlfeld, 2007), perhaps then increased placentation is an adaptation suited to meet the higher metabolic needs of foetuses in smaller mammals. This, however, may expose these foetuses to greater levels of oxidative damage, both as a result of their own metabolism, and also that transferred by the mother. Therefore, this may only be a strategy suited to r-selected species and could explain why a smaller body mass is associated with a suite of traits that define a 'fast pace of life'. If this is indeed the case, then placentation could indirectly influence life-history trait evolution, if intergenerational oxidative stress transmission is significant. Perhaps then we do find hypothetical support for Pires' life-history facilitation hypothesis (Pires *et al.*, 2011).

Indeed, our findings of a labyrinthine endotheliochorial ancestral state in eutherian mammals provide evidence that multiple selection pressures can shape placental evolution. The seemingly frequent switching between invasive hemochorial placentation and moderately invasive endotheliochorial placentation is suggestive of parent-offspring conflict. We also find that once placentation has transitioned away from greater invasion and interdigitation it does not transition back. This cannot be explained by the cooperative/altruism hypothesis but does provide evidence to support a potential protective function. Without a better explanation for this striking trend, we can suggest that it is indicative of selection

to mitigate against negative intergenerational effects. As discussed in chapter 2, protection from vertical pathogen transmission is only thought to be influenced by the degree of placental invasion, hinting at the possibility that the evolution of lower placental interdigitation is driven by a need for protection against ROS. However, this protective function ultimately cannot separate protection against vertical transmission of pathogens, from protection against vertical transmission of oxidative stress. While vertical pathogen transmission has been discussed for some time (Loke, 1982; Goldenberg, Hauth and Andrews, 2000; Benirschke, Burton and Baergen, 2012; Robbins and Bakardjiev, 2012), its contribution to intergenerational effects is largely unknown. Indeed, it certainly represents a potential avenue to perpetuate negative intergenerational consequences for species that exhibit intimate placentation. However, it is likely that the effects of vertical and horizontal pathogen transmission are also difficult to separate. Vertical transmission of pathogens across varying forms of placentation should be researched further to establish whether this has any influence on offspring survival and life-history trait evolution.

One major caveat for this comparative analysis was the largely *a priori* nature of the predictions that we made. Blue-sky thinking and hypothesis formation often lack the empirical data to allow for robust predictions. A further limitation of this study was that we did not test for interactions between placenta types. Exploring the life-history associations with placental invasiveness having controlled for interdigitation would allow us to test these interactions (Garratt, Gaillard, *et al.*, 2013). If the same association between lesser invasion and a fast pace of life (*sensu* Garratt *et al.*, 2013) held true with our larger dataset, it would certainly contradict our ‘protection hypothesis’. It is also possible that the limited range of

life history and placental traits that we considered in the present study does not provide the full picture of the evolutionary driving forces behind placental evolution. One weakness of a study such as this or Garratt *et al*'s (2013) study is the use of datasets that include life-history data for both wild and captive animals; data for captive animals are unlikely to reflect the real-life trade-offs experienced by wild counterparts, that are subject to resource limitation. This may artificially inflate the values of traits such as maximum longevity or litter size beyond what is seen naturally in the wild, which may somewhat bias the results for those analyses. However, this is not expected to have any significant influence over traits like gestation length.

The cost of reproduction

This study saw the experimental manipulation of nutrition in female banded mongooses (*Mungos mungo*) over a period of 35 months, by the supplementary feeding of egg for treatment females. We used this system to test hypotheses focussing on the oxidative cost of reproduction. The three hypotheses tested were the 'oxidative cost hypothesis' whereby increased effort in reproduction was met with an oxidative cost, the 'oxidative constraint' hypothesis, whereby reproductive effort is constrained by oxidative stress levels prior to breeding, and the 'oxidative shielding' hypothesis, where maternal oxidative damage could negatively affect offspring survival, so females downregulate their levels of oxidative stress during breeding. Markers for oxidative damage and antioxidants were collected from females during each breeding attempt, along with pre-natal investment and offspring survival.

This experimental study in a wild banded mongoose population surprisingly revealed no effect of dietary provisioning on oxidative stress levels. It was thought that supplementary feeding should alleviate some of the oxidative burden of fed individuals, thus experimentally manipulating oxidative stress (Seuss-Baum, 2007; Fletcher *et al.*, 2013; Giordano *et al.*, 2015; Nimalaratne and Wu, 2015), but this was not the case. Fed mongooses likely reduced foraging effort, or natural food availability was high during the experiment, possibly masking the effects of dietary provisioning (see Ruffino *et al.*, 2014 as an example of this). This represents the risk of using a wild population where environmental conditions and individual behaviour cannot be entirely controlled for. Disappointingly, this reduces the power of our experimental approach and thus cannot definitively identify causation beyond correlations, an unfortunate limitation to this type of study that can only be identified in hindsight. However, studies on wild populations remain an essential way of testing hypotheses based on real-life trade-offs experienced by individuals interacting with their changing environment, this cannot yet be replicated in a captive study.

Despite this limitation, we find some evidence for the oxidative cost hypothesis, where increased investment in reproduction is associated with an increase in the antioxidant glutathione. Females with high levels of antioxidant defence prior to breeding invested more heavily in reproduction, also indicating support for the oxidative constraint hypothesis. This study additionally provides tantalising support for the oxidative shielding hypothesis, whereby plasma protein carbonyls (a marker for oxidative damage) are markedly lower in pregnant females compared to non-breeders. This result, combined with a negative correlation between maternal levels of protein carbonyls and pup survival to a year of age,

suggests that protein carbonyls have a detrimental influence on offspring indicating a negative intergenerational consequence to oxidative stress.

However, a positive correlation between maternal antioxidant glutathione and pup survival to one year of age suggests a need to mitigate against this negative intergenerational effect. Interestingly, in this study, we observe the oxidative cost and constraint hypotheses via antioxidant defence, rather than in markers of oxidative damage. This too suggests a desire to mitigate against oxidative assaults rather than suffer the consequences. If natural food availability were high throughout the experiment, this could explain why we see the effects portrayed in antioxidant defence rather than via damage levels. It is worth noting that individuals do not adjust their levels of oxidative stress in relation to their baseline oxidative state, perhaps this is too costly. These findings indicate that oxidative stress is an important part of reproduction which can lead to negative intergenerational consequences. By tailoring their investment in reproduction to their baseline oxidative state, or lowering oxidative damage and increasing antioxidant defence, individuals and their offspring may avoid high levels of damage as a consequence of breeding. Oxidative constraint and oxidative shielding are likely to be co-occurring mechanisms that optimise lifetime reproductive success in response to an oxidative cost of reproduction.

Synthesis

In theory, the intergenerational cost of reproduction may vary by placenta type if placentation is responsible for the control of maternal resources. Parent-offspring conflict remains a popular explanation for placental evolution (Haig, 1993; Crespi

and Semeniuk, 2004; Pires, McBride and Reznick, 2007; Banet, Au and Reznick, 2010; Capellini, Venditti and Barton, 2011; Garratt, Gaillard, *et al.*, 2013). Haig suggested that the degree of placental invasion may indicate whether mother or offspring have greater control over maternal resources during gestation (Haig, 1993). Indeed, placentation has been hypothesised as an adaptive reproductive solution under high resource availability, but a risky strategy if resources are limited (Trexler and DeAngelis, 2003; Pires, McBride and Reznick, 2007). Under conditions of limited resources, placental female *Poeciliopsis* fish have been observed to sacrifice body condition to sustain young (Banet and Reznick, 2008; Banet, Au and Reznick, 2010). This could theoretically reduce their lifespan (Trexler and DeAngelis, 2003), while non-placental species maintain body condition at the expense of reproduction (Banet and Reznick, 2008; Banet, Au and Reznick, 2010). In low resource conditions, placental females also produce offspring of lower quality which detrimentally affects their survival, lowering the mother's fitness. If the mother was fully in charge of her resource allocation, under low resource conditions she should abort some embryos to increase the viability of the others (Banet, Au and Reznick, 2010), yet we do not see evidence for this (Banet and Reznick, 2008; Banet, Au and Reznick, 2010). Therefore, under such conditions, we see the potential for intergenerational effects to perpetuate as a consequence of foetal control of maternal resources. The degree of maternal exploitation by the foetus may also be incurred as an oxidative cost to the mother, potentially the foetus sacrifices greater oxidative protection for greater access to resources.

As with all carnivores except hyenas, mongooses exhibit moderately invasive endotheliochorial placentation (Elliot and Crespi, 2008). While we were unable to

confirm the placental interdigitation of mongooses specifically, all carnivores with data for interdigitation within the dataset for chapter 2, exhibit labyrinthine placentation, as well as the closely related meerkat, *Suricata suricatta* (Benirschke, 2006). Therefore, it is likely that the banded mongoose shares this trait with its fellow Carnivores. Banded mongooses, coincidentally, make an interesting choice of study species for chapter 3, as they likely display both ancestral placental traits of labyrinthine endotheliochorial placentation. Furthermore, the ancestral state reconstructions in chapter 2 (Figures 11 and 13) imply that these traits are actually retained rather than reverted back to their ancestral state. Therefore, all Carnivores (except hyenas which exhibit hemochorial placentation), alongside the order Scandentia, retain the ancestral placental traits. Such species would be interesting to study to explore selection pressures acting on ancestral mammals enabling them to retain their ancestral placentation and hint at the selection pressures that may drive their diversification. Indeed, mechanisms such as oxidative shielding (evidence of which has been found in banded mongooses-see chapter 3 and Vitikainen *et al.* (2016) may provide a means to mitigate against intergenerational oxidative damage without the need to decrease placental invasion or interdigitation. This poses an interesting question; does oxidative shielding vary across different placental forms? If intergenerational damage poses less of a risk for species exhibiting a faster pace of life and high placental invasion and interdigitation, then we might expect these species to invest less in oxidative shielding. Conversely, if protection from vertical transmission of pathogens drives the evolution of decreased placental invasion (and reduced foetal exposure to oxidative stress are a subsequent by-product of that), then we could potentially expect species with high levels of placental invasion to invest more heavily in oxidative shielding

to mitigate against the effects of increased exposure to oxidative stress. Answering this question may help us to disentangle the driving forces behind the protective role of placentation.

Unsurprisingly, human placentation remains the best-studied system regarding the implications of oxidative stress, thanks to the large investment in medical research. It was previously assumed that the human placenta originated to supply as much oxygen to the foetus as possible, this is largely true during the second half of pregnancy, but not in the first half (Jauniaux, Poston and Burton, 2006). During gestation, early development takes place in a low oxygen (O_2) environment. Jauniaux, Gulbis and Burton developed the hypothesis that the placenta limits, rather than facilitates, O_2 supply to the foetus during organogenesis in humans (Jauniaux, Gulbis and Burton, 2003); this perhaps could represent some form of oxidative shielding. In most species, placental attachment to the uterine tissue occurs after organogenesis is complete and when embryonic development is quite advanced, ensuring that early development takes place in a low oxygen environment. Placental and embryonic cells are very sensitive to oxidative stress because of their extensive cell division and associated exposure of their DNA (Burton, et al., 2003). This physiological hypoxia during the most significant part of foetal development provides some protection from deleterious and teratogenic effects of ROS (Jauniaux, Poston and Burton, 2006), and is also necessary to maintain stem cells in a fully pluripotent state (Ezashi, Das and Roberts, 2005; Jauniaux, Poston and Burton, 2006). Naturally, oxidative stress in humans has been linked to a variety of severe pregnancy conditions including preeclampsia and miscarriage, intrauterine growth restriction, preterm labour and spontaneous abortion (Jauniaux, Poston

and Burton, 2006; Hoffmann *et al.*, 2008; Burton and Jauniaux, 2011), while these particular conditions are seemingly rare in other mammalian species (Jauniaux, Poston and Burton, 2006). However, research shows that some oxidative stress is vital to foetal development, triggering the formation of foetal membranes and trophoblastic proteins and stimulating the upregulation of ROS defence (Jauniaux *et al.*, 2003; Jauniaux, Poston and Burton, 2006). Humans represent a species that exhibits a slow pace of life while having surprisingly highly invasive hemochorial placentation. They also face, at least in the first world, almost no limitation to resources, which possibly enables them to sidestep the trade-off between reproduction and survival, allowing them to live far longer life spans than would be expected for their size. Perhaps then, the villous (low degree of) interdigitation of humans enables them to sustain this evolutionary strategy by protecting against some oxidative assaults during foetal development. Alternatively, perhaps, conditions such as preeclampsia and miscarriage represent a maladaptation to the fine-tuned, but constantly changing oxygen environment as a consequence of our artificially increased lifespan (Jauniaux, Poston and Burton, 2006).

Avenues for future study

The poeciliid genus of fish provide an exciting opportunity to explore factors driving placental evolution, with around 20 species in this genus, extensive placentotrophy has evolved independently at least three times and species exhibit varying degrees of matrotrophy (Reznick, Mateos and Springer, 2002). Indeed, this system has been used by others for the same purpose (Reznick, Mateos and Springer, 2002; Pires, McBride and Reznick, 2007; Banet and

Reznick, 2008; Banet, Au and Reznick, 2010; Pires *et al.*, 2011; Hagmayer *et al.*, 2020). Of course, matrotrophy index (MI), which is used to measure placentation in these fish, is not necessarily synonymous with the placental invasion or interdigitation that we see in mammals. However, it could still provide useful information on the possible consequences of varying the extent of maternal provisioning after fertilisation.

Much like the banded mongoose study in chapter 3, we could attempt to experimentally manipulate oxidative stress in mothers of species exhibiting varying degrees of placentation using a supplementary feeding experiment, and monitoring life-history traits and survival of the young. This would allow for a repeat of the dietary provisioning experiment in chapter 3, but conducted in a laboratory setting to keep environmental conditions consistent, thus mitigating against any issues faced in a wild system. Although it has not been tried in poeciliid fish, a recent study highlights a technique that uses fluorescent markers to measure oxidative stress in live zebrafish larvae in near-real time (Mourabit *et al.*, 2019). Cell lines of *Poeciliopsis lucida* hepatoma have also previously been used to study the influence of oxidative stress at a cellular level (Choi and Oris, 2000, 2003; Rau *et al.*, 2004; Puerto *et al.*, 2009; Selvaraj, Yeager-Armstead and Murray, 2012; Pérez-Albaladejo, Solé and Porte, 2020). Research studying the effects of oxidative stress have also been explored and discussed in other fish species (Lackner, 1998; Stoliar and Lushchak, 2012; Lushchak, 2016; Bacchetta *et al.*, 2017; Wu *et al.*, 2021), so theoretically measuring oxidative stress markers in poeciliid fish is possible, although there may be limitations using smaller species like poecilids that you would not otherwise have with larger species (e.g. the amount of blood that can be safely drawn) (Lushchak, 2016). To overcome

these potential limitations, you could use a cross-sectional approach, or perform supplementary feeding experiments to see whether dietary provisioning can influence the oxidative state of poeciliid fish. Equally, these poeciliid fish could be used for studies on vertical pathogen transmission as a function of placentotrophy, if a suitable pathogen can be identified. Here you have to potential to explore whether infected species with higher MI are more likely to vertically transmit pathogens and whether this has a detrimental impact on offspring survival or influences life-history traits.

Conclusion

This thesis provides evidence that oxidative stress can have negative intergenerational consequences in mammals that may be mitigated in a variety of ways. Namely, for each breeding attempt, an individual may either constrain its reproductive investment in response to its baseline oxidative state or invest in oxidative shielding to minimise the exposure of its vulnerable offspring *in utero*. Over time, a species may evolve lower degrees of placental invasion and interdigitation as a means of further protecting offspring from negative intergenerational effects, either in conjunction with these previous mechanisms or instead of them, potentially allowing species to maintain their ancestral placental traits or facilitating their metabolic demands. This research provides a compelling argument for the oxidative cost of reproduction and emphasizes the need for mothers to mitigate against the detrimental consequences for the sake of their offspring and their own fitness. This thesis also highlights the importance of including placentation in future studies exploring life-history evolution and provides a promising new avenue for further studying intergenerational effects in

mammals. Further research is needed to separate the role of protection in placentation by mitigating against vertical pathogen transmission or transmission of oxidative stress; the poeciliid fish system may provide an excellent opportunity to test these ideas experimentally. Ultimately, placentation may be fundamentally important to the transmission of intergenerational effects and have the potential to shape life-history evolution. The abundance of life-history strategies we see across mammals reflects the complex web of factors driving its evolution and could likely explain the wonderful diversity of mammalian placentation we see today.

Appendix A

Supplementary Information for Chapter 2

Placentation breakdown

Table S1: Number of species by Placental Invasion and Placental Interdigitation, where grey cells indicate that no species within the dataset displayed these placental traits simultaneously.

		Placental Interface		
		Epitheliochorial	**Endotheliochorial**	**Hemochorial**
Placental Interdigitation	**Villous**	85	0	6
	Trabecular	33	0	37
	Labyrinthine	0	62	110

Body mass residual plots

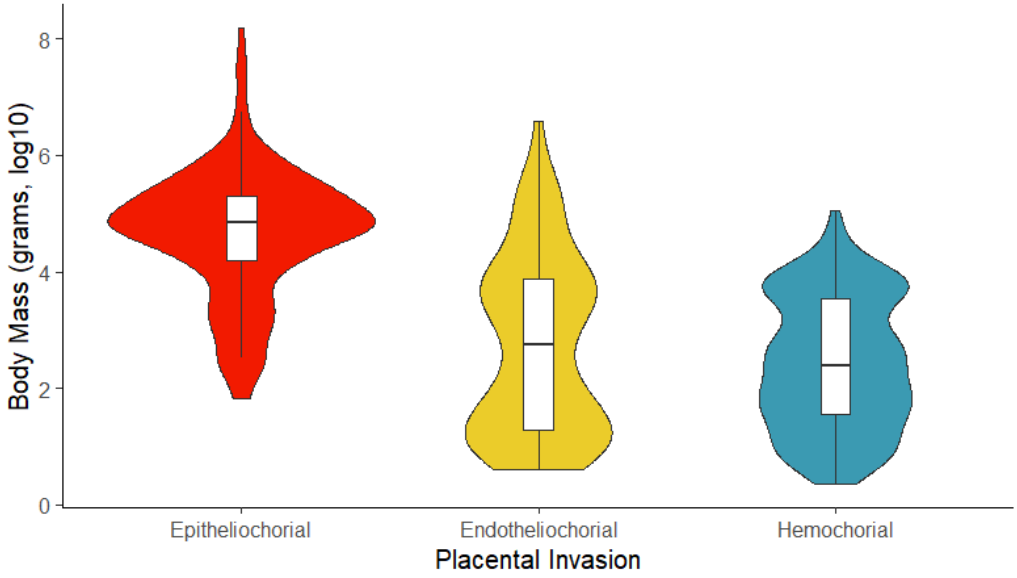

Figure S1: Raw relationship between placental invasion and body mass. Red violins correspond to the least invasive epitheliochorial placenta, yellow violins correspond to the mid-invasive endotheliochorial placenta and blue violins correspond to the most invasive hemochorial placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

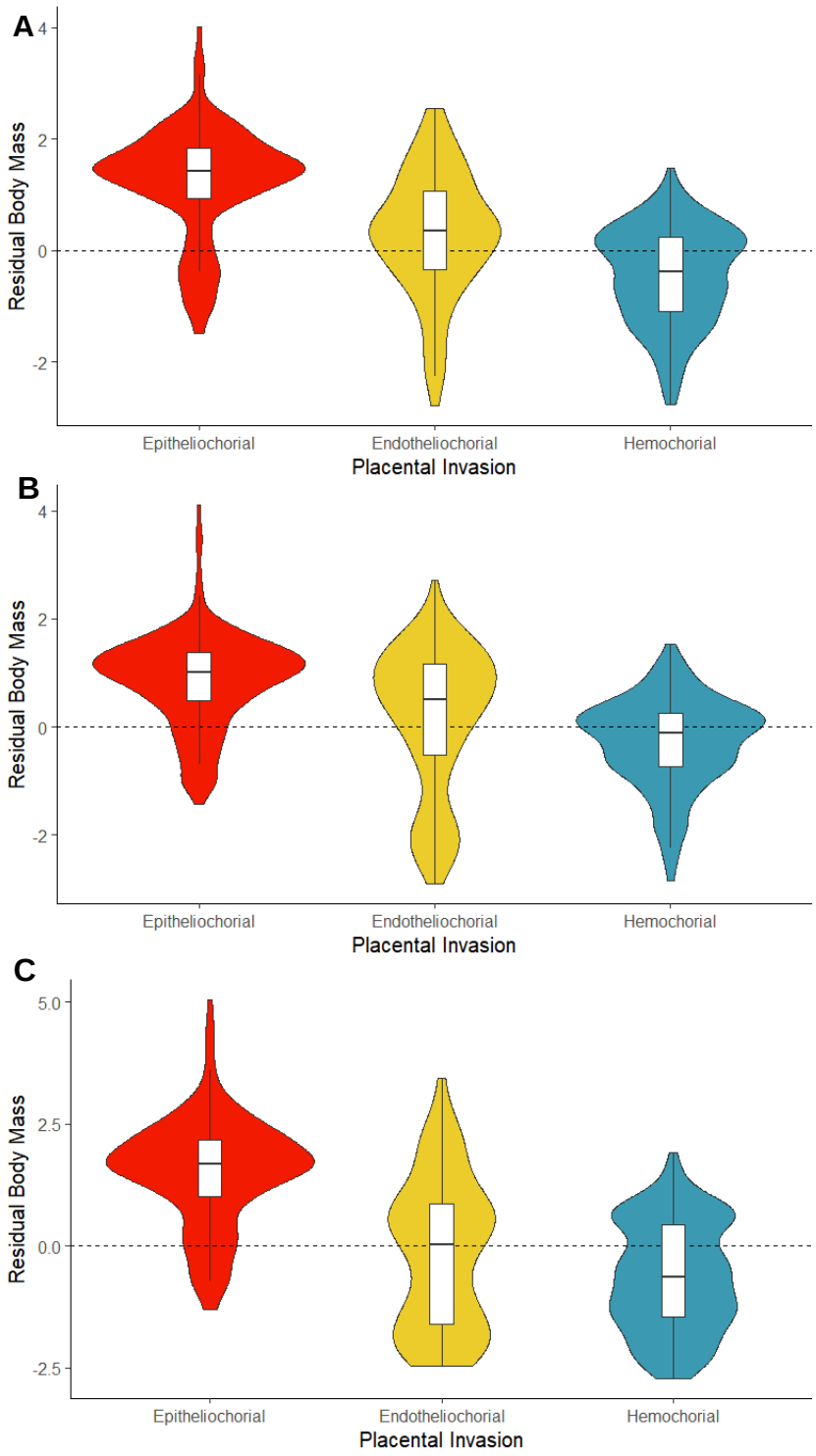

Figure S2: PGLS model residual body mass where life-history traits and phylogeny have been controlled for, plotted against placental invasiveness. Panel A shows the models controlled for maximum longevity, B for gestation length and C for litter size. Red violins correspond to the least invasive epitheliochorial placenta, yellow violins correspond to the mid-invasive endotheliochorial placenta and blue violins correspond to the most invasive hemochorial placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

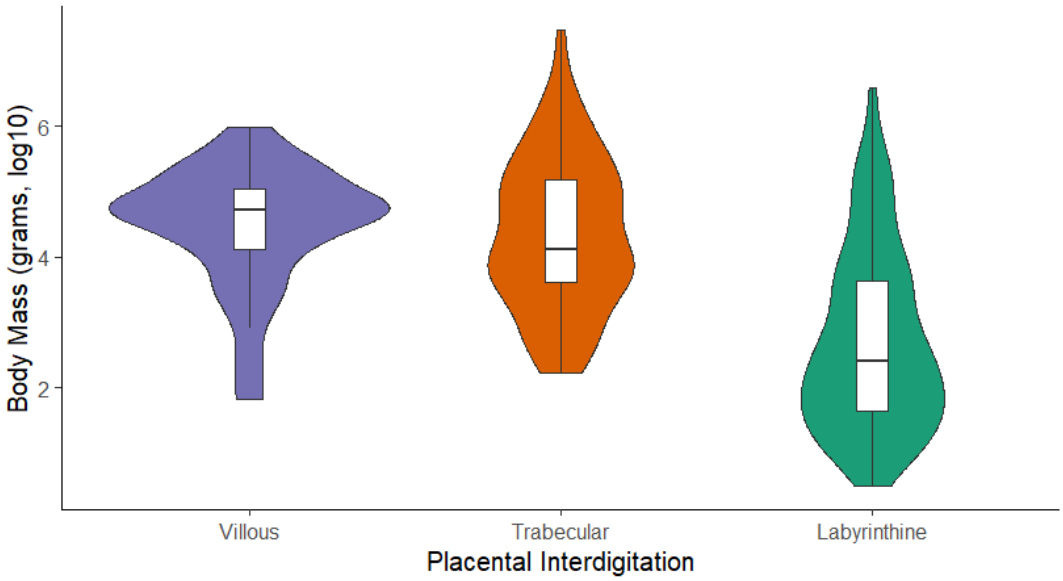

Figure S3: Raw relationship between placental interdigitation and body mass. Purple violins correspond to the least interdigitated villous placenta, orange violins correspond to the mid-interdigitated trabecular placenta and green violins correspond to the most interdigitated labyrinthine placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

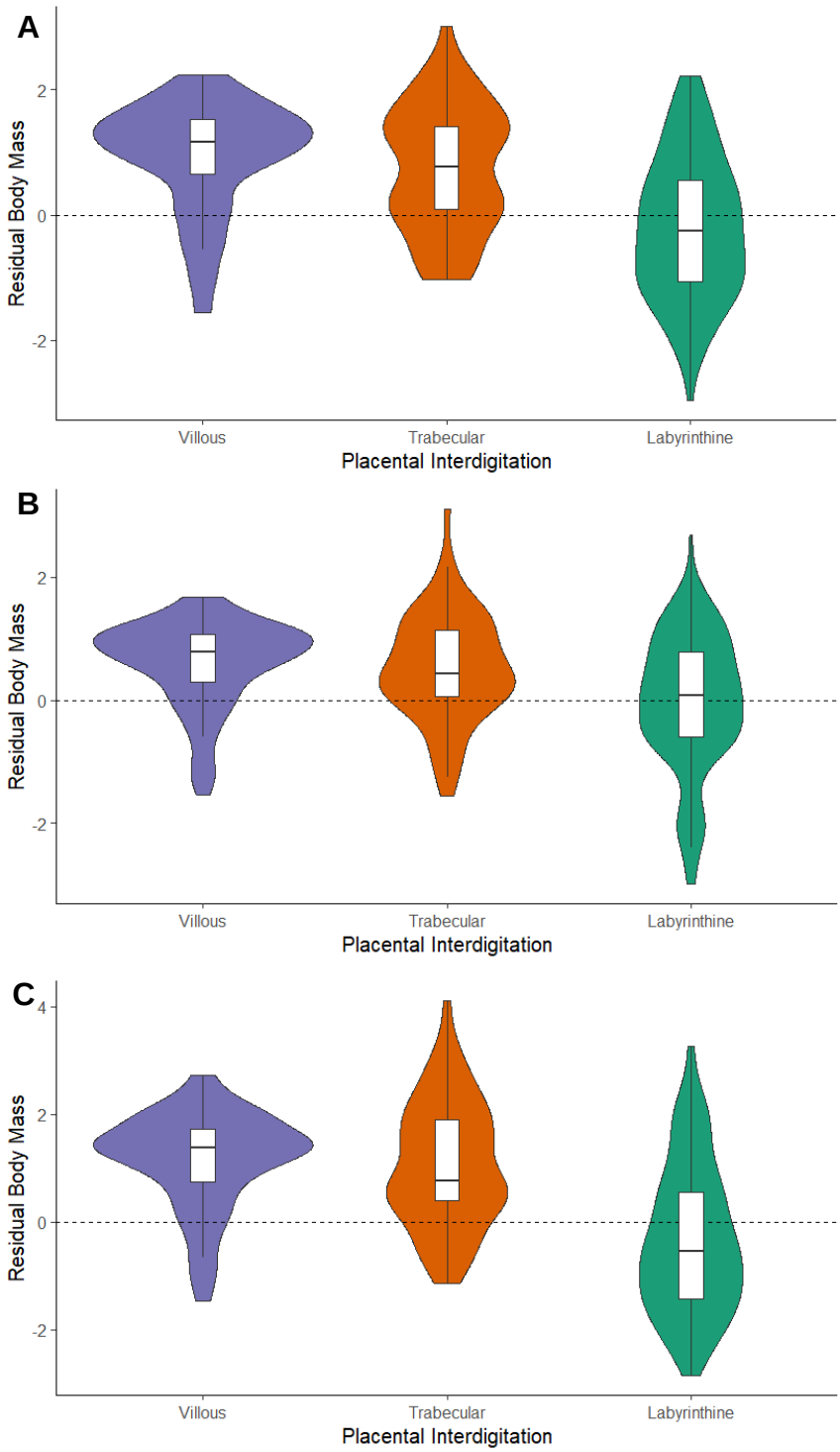

Figure S4: PGLS model residual body mass where life-history traits and phylogeny have been controlled for, plotted against placental interdigitation. Panel A shows the models controlled for maximum longevity, B for gestation length and C for litter size. Purple violins correspond to the least interdigitated villous placenta, orange violins correspond to the mid-interdigitated trabecular placenta and green violins correspond to the most interdigitated labyrinthine placenta type. Boxplots display the median and inter-quartile range for each category, outliers have been removed.

Alternative method for calculating lambda

When looking at associations between placental interdigitation and body mass whilst controlling for phylogeny and maximum longevity (see results marked with an * in table 4 of the main text), the standard maximum likelihood approach for calculating lambda using the PGLS method from the “caper” package (Orme *et al.*, 2018) gave an error. Instead, lambda was estimated by looking at the maximum likelihood surface, choosing the value of lambda that gave the highest log likelihood (see figure S5). This selected value for lambda was used in the model to provide the results for the model estimates, standard error, and significance.

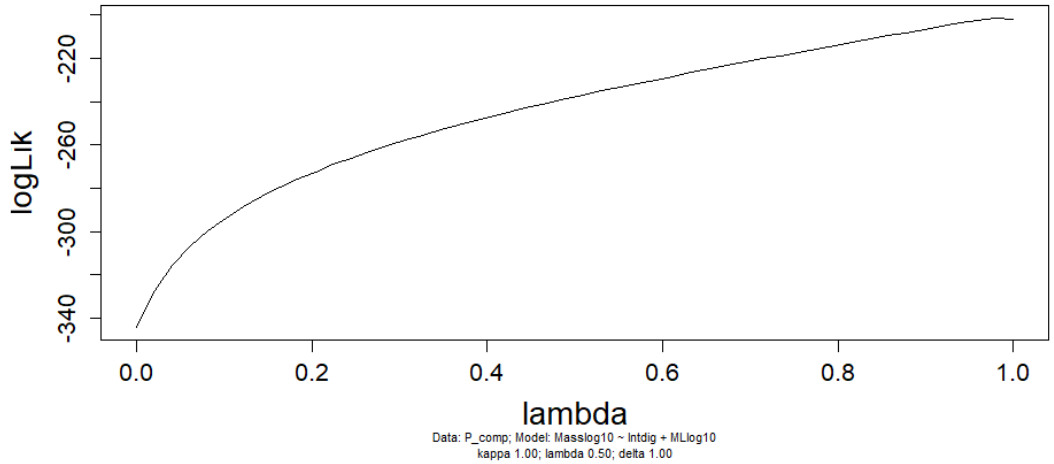

Figure S5: The likelihood surface for lambda where body mass is the response variable, and interdigitation and maximum longevity are the explanatory variables. Lambda indicates the phylogenetic signal and logLik is the log-likelihood. The text under the x-axis shows the details of the model used, where body mass and maximum longevity are transformed using the $\log_{10}$ transformation. P_comp is the name of the dataset used, where the phylogenetic tree is combined with the database to ensure a consistent structure. Kappa and delta indicate gradual evolution and are set to one for simplicity. Lambda =0.50 represents the starting value of lambda used in the model to generate the likelihood surface.

Reconstruction of ancestral state using the ER model

Reconstructing the ancestral state of placental invasion using the equal rates model predicts an ancestral state of hemochorial invasion (figure S6).

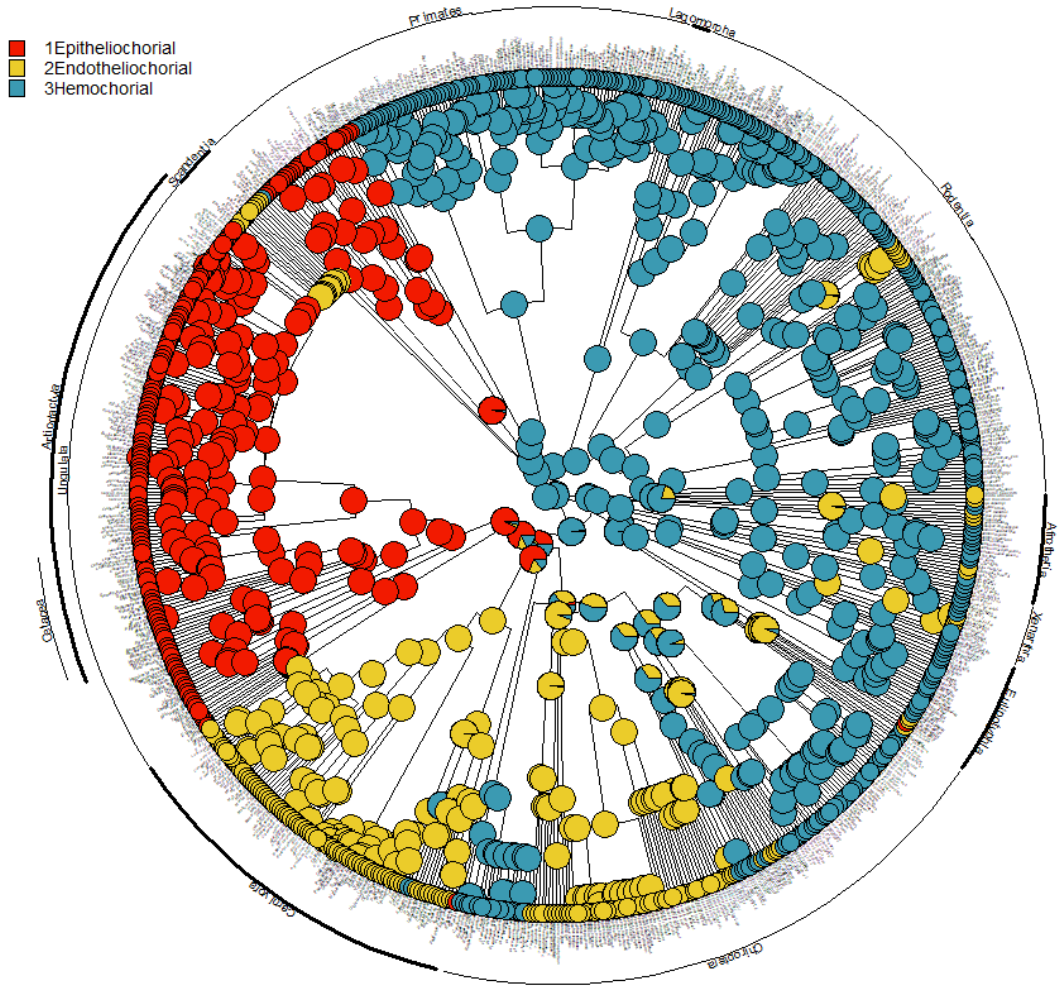

Figure S6: The Equal Rates Model (ER) placental invasion, The likelihood of each placental trait at each node is displayed as a mini pie chart, where red indicates the least invasive epitheliochorial placenta, yellow is the moderately invasive endotheliochorial placenta and blue is the most invasive hemochorial placenta. The node at the centre represents the common ancestor of eutherian mammals, and nodes at the outer edge represent the extant species labelled in the phylogeny. Eutherian mammalian clades are labelled around the outside of the tree.

The ancestral state reconstruction using the equal rates model shows indicate that labyrinthine interdigitation was ancestral. This same result as the ordered model provides strong evidence that labyrinthine interdigitation is almost certainly ancestral (figure S7).

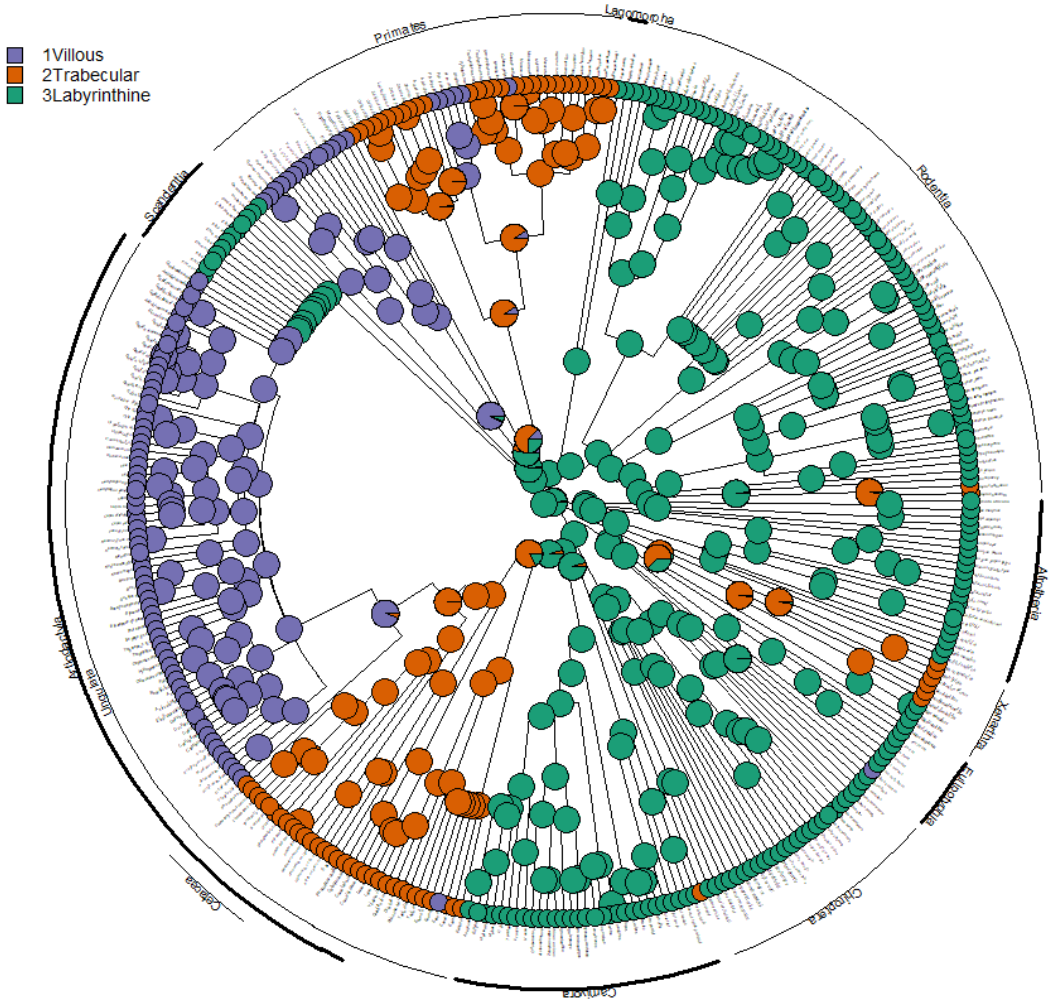

Figure S7: The Equal Rates Model (ER) for placental interdigitation, The likelihood of each placental trait at each node is displayed as a mini pie chart, where purple indicates the villous placenta lowest degree of interdigitation, orange is the trabecular placenta and green is the labyrinthine placenta with the highest degree of interdigitation. The node at the centre represents the common ancestor of eutherian mammals, and nodes at the edge represent the extant species labelled in the phylogeny. Eutherian mammalian clades are labelled around the outside of the tree.

Phylogenetic distribution of life-histories

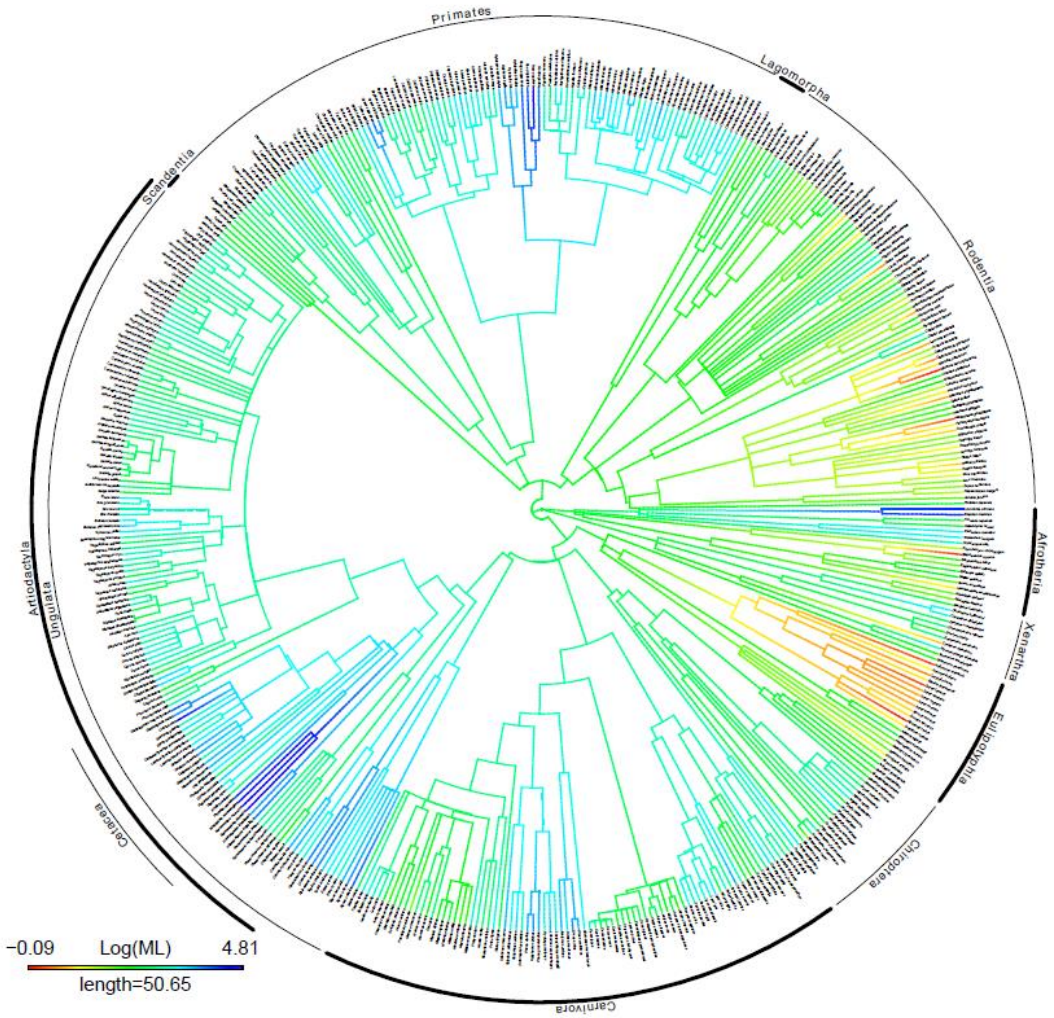

Figure S8: log maximum longevity plotted onto the mammalian phylogeny, where the colour scale from red to blue indicates short to long lifespans, respectively. The node at the centre represents the common ancestor of eutherian mammals, and nodes at the outer edge represent the extant species labelled in the phylogeny. Eutherian mammalian clades are labelled around the outside of the tree.

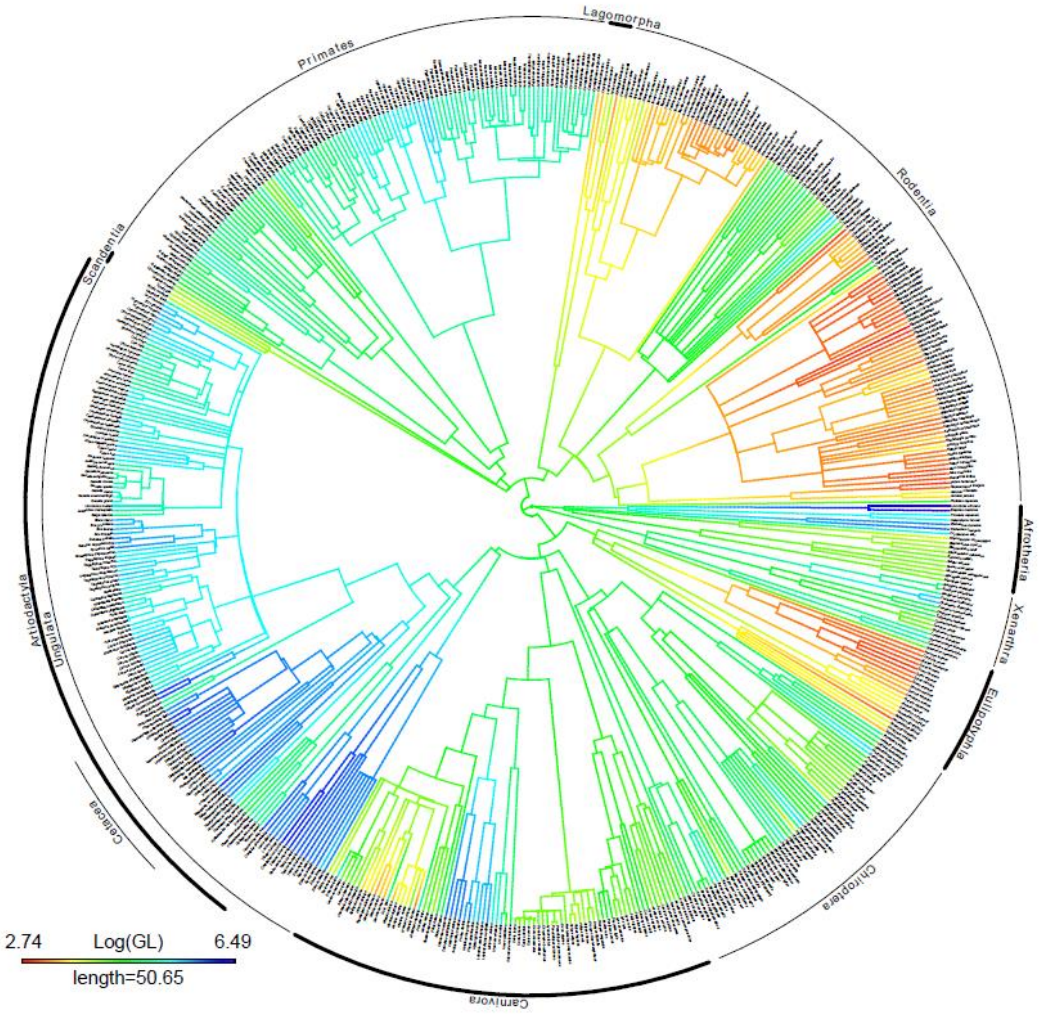

Figure S9: log gestation length plotted onto the mammalian phylogeny, where the colour scale from red to blue indicates short to long gestation lengths, respectively. The node at the centre represents the common ancestor of eutherian mammals, and nodes at the outer edge represent the extant species labelled in the phylogeny. Eutherian mammalian clades are labelled around the outside of the tree.

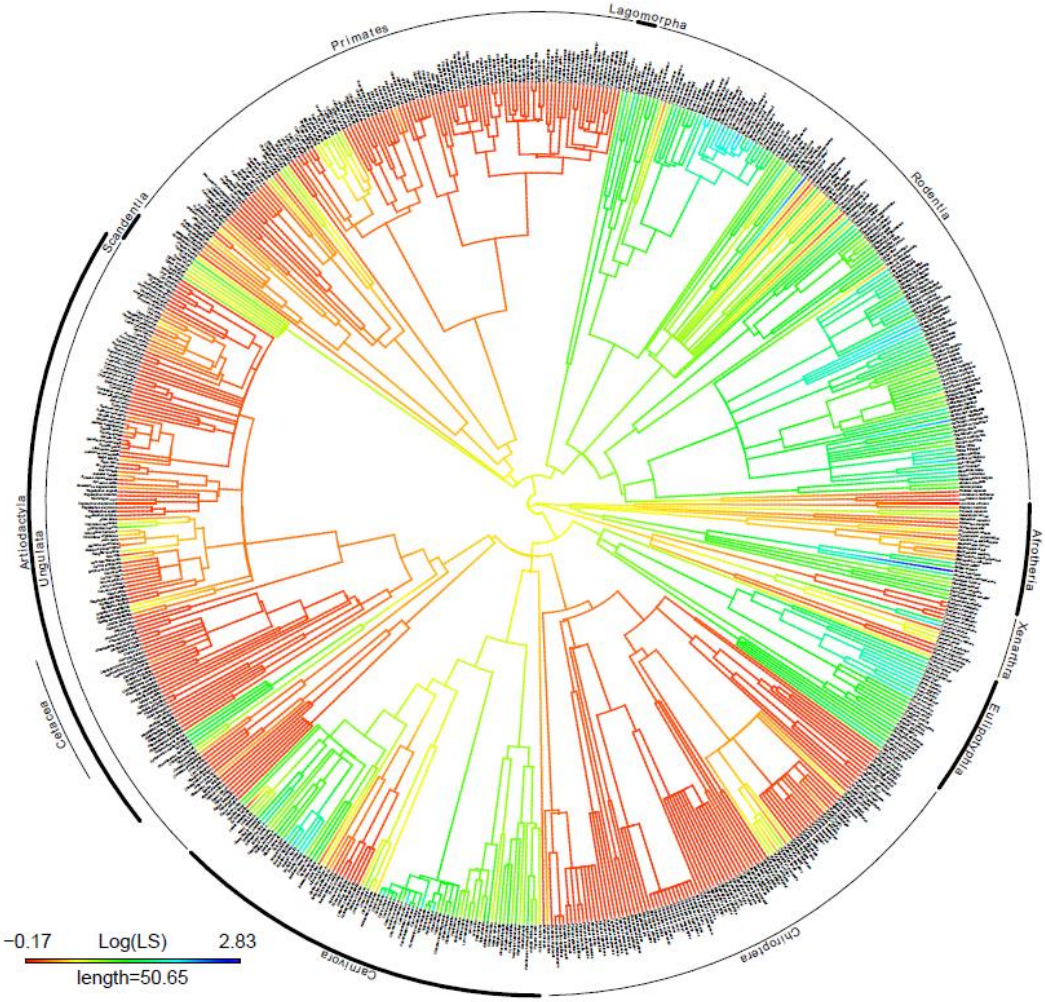

Figure S10: log litter size plotted onto the mammalian phylogeny, where the colour scale from red to blue indicates small to large litter sizes, respectively. The node at the centre represents the common ancestor of eutherian mammals, and nodes at the outer edge represent the extant species labelled in the phylogeny. Eutherian mammalian clades are labelled around the outside of the tree.

Database description

This data set comprises 732 species of eutherian mammal, from 21 different orders according to Walker's mammals of the world (Nowak, 1991). This dataset is built from a variety of placental and life-history traits.

The placental traits in this database are placental interface and interdigitation. The main life-history traits include body mass, mating system, maximum longevity, gestation length and litter size, taken from the PanTHERIA dataset (Jones *et al.*, 2009). It was built around available data for placental interface in accordance with the phylogenetic tree produced by (Bininda-Emonds *et al.*, 2007), so 100% of species (all 732) listed in this data set have data for placental invasion.

Life-history traits such as body mass, gestation length and litter size have a good representation in the data set across the mammalian orders, 98.09% (719spp), 77.35% (567spp) and 90.45% (663spp) respectively. These traits also had at least one entry for every mammalian order within the data set.

63.8% of species in the database have data for Maximum longevity (467spp). Both orders Pholidota and Dermoptera have no data for maximum longevity, and orders Chiroptera and Scandentia are underrepresented.

However, mating system and interdigitation are very underrepresented within this dataset, with less than half of the species having data for either trait. Interdigitation has records for 333 species, which makes up 45.43% of species recorded. Mammalian orders underrepresented for (less than 50% of species in that order that have data points) interdigitation are Carnivora, Cetacea, Chiroptera, Cingulata, Erinaceomorpha, Primates, Rodentia and Soricomorpha. Whilst some orders are underrepresented proportionally, all orders recorded here have at least one species with interdigitation data.

Only 340 species in this dataset have information on mating system (46.38%). Orders underrepresented (less than 50% of species in that order that have data points) for mating system are Afrosoricida, Cetacea, Chiroptera, Cingulata, Dermoptera, Erinaceoomrpha, Pholidota, Pilosa, Rodentia, Scandentia, Soricomorpha and Tubulidentata. Orders with no representation for mating system at all are Dermoptera, Pholidota and Tubulidentata, although it should be worth noting that for each of these orders, only one species is actually recorded in the dataset.

Of the 732 species in the dataset, 316 species were identified as having species-specific reports identifying placental invasion, 43 species had references that were at least genus-specific, and 373 species were most likely assumptions based on closely related species. Of the 333 species with interdigitation data, 269 were identified as having a species-specific report identifying interdigitation type, 35 were at least genus-specific and 29 were likely assumptions.

Appendix B

Supplementary Information for Chapter 3

Ultrasound scanning

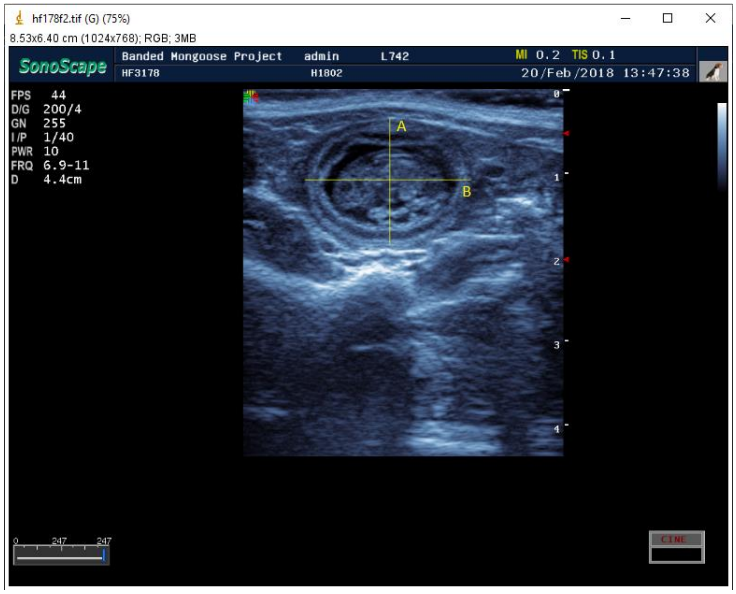

Figure S1: Foetal ultrasound image. The elliptical shape measured using yellow perpendicular lines 'A' and 'B' makes up the gestational sac. Measurements 'A' and 'B' correspond to the terms in the formula for cross-sectional area.

Quantification of malondialdehyde (MDA)

Plasma malondialdehyde was determined using high-performance liquid chromatography (HPLC) with fluorescence detection following (Nussey *et al.*, 2009) with some modifications described here. All chemicals were HPLC grade, and chemical solutions were prepared using ultrapure water (Milli-Q Synthesis; Millipore, Watford, UK). Briefly, 20 µl of plasma or standard (1,1,3,3-tetraethoxypropane, TEP; see below), 20 µl 0.05% (w/v) butylated hydroxytoluene solution in 95% ethanol, and 160 µl of 0.44M phosphoric acid

solution were added to a 2ml screw cap reaction tube. To initiate the reaction, 40 µl of 42mM 2- thiobarbituric acid (TBA) was added. Tubes were then capped and briefly vortexed, before being incubated on a dry heat block for 1 hour at 100°C to allow the formation of MDA-TBA adducts. After the incubation period, samples were placed on ice for five minutes to stop any further reaction. nButanol (160 µl) was added, and tubes were vortexed for 20 seconds. Samples were then centrifuged at 12,000 x g for 3 min at 4°C. A 100 µl aliquot of the upper butanol phase was carefully transferred to a 0.3ml crimp top HPLC vial. Samples (40 µl) were injected into an Agilent 1200 series HPLC system (Agilent Technologies, California, USA) fitted with Thermo Scientific Hypersil 5µ ODS 100 x 4.6mm column (Patr no. 30105-104630) The mobile phase was methanol-buffer (40:60, v/v), the buffer being a 50mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5M potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 ml min-1. The column oven was set at 37°C. Peaks were collected using a fluorescence detector (Agilent G1321C), with excitation and emission wavelengths of 515nm and 553nm, respectively. Peaks were quantified relative to an external calibration curve prepared using a TEP stock solution (5µM in 40% ethanol) serially diluted using 40% ethanol to give known values in the range 0 – 5 µM.

Table S1: Test of prediction 1.1. Linear mixed model exploring the link between oxidative stress markers and provisioning treatment, stage of reproduction, and their interaction in pregnant females. P-values highlighted in bold do not remain significant after correction using the false discovery rate procedure.

		Protein Carbonyl				**MDA**				**SOD**				**Glutathione**			
		N	Estimate +/- se	F-value DF	P-value	N	Estimate +/- se	F-value DF	P-value	N	Estimate +/- se	F-value DF	P-value	N	Estimate +/- se	F-value DF	P-value
Fixed Effects	Intercept	264	0.18+/-0.18			293	-0.00+/-0.16			240	0.13+/-0.17			259	0.04+/-0.18		
	Provisioning treatment (Provisioned)		0.06+/-0.23	0.12 $_{1,12.6}$	0.73		0.14+/-0.18	1.72 $_{1,265}$	0.19		-0.29+/-0.20	2.09 $_{1,222}$	0.15		-0.06+/-0.23	0.26 $_{1,9.7}$	0.62
	Stage of reproduction			**3.82 $_{2,207}$**	**0.02**			0.18 $_{2,262}$	0.84			1.34 $_{2,218}$	0.26			0.83 $_{2,234}$	0.44
	(Pregnancy)		-0.13+/- 0.18				0.03+/-0.18				-0.37+/-0.19				-0.01+/-0.20		
	(Lactation)		0.26+/- 0.19				0.05+/-0.19				-0.21+/-0.20				-0.03+/-0.21		
	Provisioning treatment x Stage of reproduction			0.42 $_{2,200.1}$	0.66			0.05 $_{2,241}$	0.95			0.78 $_{2,207}$	0.46			0.77 $_{2,212}$	0.47
	(Provisioned x Pregnancy)		-0.14 +/-0.24				-0.04+/-0.24				0.30+/-0.26				-0.19+/-0.27		
	(Provisioned x Lactation)		-0.23+/-0.25				0.04+/-0.25				0.04+/-0.27				0.16+/-0.28		
Random Effects			*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*
	Mother ID		*0.10*	*0.32*	*9.71*		*0.00*	*0.00*	*0.00*		*0.00*	*0.00*	*0.00*		*0.08*	*0.28*	*7.84*
	Litter ID		*0.31*	*0.56*	*30.10*		*0.36*	*0.60*	*33.64*		*0.32*	*0.57*	*32.99*		*0.19*	*0.43*	*18.63*
	Residual		*0.62*	*0.79*	*60.19*		*0.71*	*0.84*	*66.36*		*0.65*	*0.81*	*67.01*		*0.75*	*0.86*	*73.53*

Table S2: Test of prediction 1.2. Linear mixed model exploring the link between maternal investment/offspring survival and provisioning treatment, stage of reproduction, and their interaction in pregnant females. P-values highlighted in bold do not remain significant after correction using the false discovery rate procedure.

		Pre-natal investment				**Offspring' body mass at emergence**				**Survival to emergence**				**Survival to 12 months**			
		N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	Chisq	P-value
Fixed effects	Intercept	24	0.37+/-0.06			92	146.03+/-52.88			30	0.36+/-0.66			108			
	Offspring age at emergence		-	-	-		2.89+/-1.15	**6.24** $_{1,56.3}$	**0.02**		-	-	-		-	-	-
	Foetus number		-	-	-		-	-	-		0.54+/-0.23	**5.46** $_{1,26}$	**0.03**		-	-	-
	Provisioning treatment (Provisioned)		0.04+/-0.05	0.69 $_{1,14.3}$	0.42		10.22 +/-14.90	0.46 $_{1,11.6}$	0.51		0.29+/-1.13	0.07 $_{1,26}$	0.80		-0.28+/-0.37	0.58	0.44
	Provisioning treatment x Foetus number (Provisioned x Foetus number)		-	-	-		-	-	-		-0.30+/-0.40	0.58 $_{1,26}$	0.45		-	-	-
Random Effects			*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*
	Mother ID		*<0.01*	*<0.010.*	*<0.01*		*301.90*	*17.37*	*7.61*		*0.00*	*0.00*	*0*		*<0.01*	*0.02*	-
	Litter ID		*0.02*	*0.15*	*73.11*		*1541.90*	*39.27*	*38.86*		*0.00*	*0.00*	*0*		*0.95*	*0.98*	-
	Residual		*<0.01*	*0.09*	*26.89*		*2124.30*	*46.09*	*53.53*		*0.54*	*0.73*	*100*		-	-	-

Table S3: Test of the oxidative cost hypothesis: prediction 2.1. Linear mixed model exploring the link between intra-individual changes in oxidative status and reproductive investment. P-values highlighted in bold do not remain significant after correction using the false discovery rate procedure. M1 indicates model 1 and M2 indicates model 2.

	Change in PC				**Change in MDA**				**Change in SOD**				**Change in Glutathione**			
	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value
M1 Intercept	18	-0.48+/-0.02			18	-0.08+/-0.34			16	0.05+/-0.52			18	-0.17+/-0.20		
M1 Pre-natal investment		0.24 +/-0.24	0.96 $_{1,16}$	0.34		0.51+/-0.27	3.59 $_{1,13.4}$	0.08		0.42+/-0.40	1.09 $_{1,13.9}$	0.31		**0.53+/-0.26**	**4.25 $_{1,16}$**	**0.05**
M2 Intercept	42	-0.20+/-0.27			42	0.13+/-0.30			41	-0.07+/-0.29			38	-0.05+/-0.32		
M2 Offspring body mass at emergence corrected		0.05+/-0.16	0.11 $_{1,29.9}$	0.74		0.08+/-0.11	0.53 $_{1,28.9}$	0.47		0.03+/-0.06	0.20 $_{1,29.1}$	0.66		0.04+/-0.07	0.37 $_{1,21.7}$	0.55
		Variance	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*
M1 Mother ID random effect		*0.00*	*0.00*	*0.00*		*0.36*	*0.60*	*35.64*		*0.00*	*0.00*	*0.00*		*0.00*	*0.00*	*0.00*
M1 Litter ID random effect		*0.00*	*0.00*	*0.00*		*0.56*	*0.75*	*55.45*		*1.34*	*1.16*	*76.14*		*0.00*	*0.00*	*0.00*
M1 Residual random effect		*0.65*	*0.80*	*100*		*0.09*	*0.30*	*8.91*		*0.42*	*0.65*	*23.86*		*0.74*	*0.86*	*100*
M2 Mother ID random effect		*0.46*	*0.68*	*42.59*		*0.06*	*0.24*	*0.48*		*0.02*	*0.13*	*1.59*		*0.31*	*0.56*	*24.80*
M2 Litter ID random effect		*0.19*	*0.43*	*17.59*		*0.89*	*0.95*	*77.22*		*0.92*	*0.96*	*88.89*		*0.86*	*0.93*	*68.80*
M2 Residual random effect		*0.43*	*0.65*	*39.81*		*0.20*	*0.45*	*17.66*		*0.10*	*0.31*	*9.52*		*0.08*	*0.28*	*6.40*

Table S4: Test of the oxidative constraint hypothesis: prediction 2.2. Models exploring the link between maternal investment and oxidative stress markers before pregnancy, with the relevant covariates. P-values highlighted in bold do not remain significant after correction using the false discovery rate procedure

		Pre-natal investment				**Offspring' body mass at emergence**			
		N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value
Fixed Effects	Intercept	16	0.36+/-0.05			63	96.80+/-66.40		
	Offspring age at emergence		-	-	-		**3.86 +/- 1.50**	**6.65** $_{1,51.7}$	**0.01**
	PC		0.07 +/- 0.03	4.50$_{1,7.4}$	0.07		-1.27 +/- 7.27	0.03 $_{1,41.1}$	0.86
	MDA		-0.01 +/- 0.04	0.06 $_{1,10.7}$	0.80		5.83 +/- 6.31	0.85 $_{1,54.6}$	0.36
	SOD		0.01 +/- 0.02	0.20 $_{1,7.37}$	0.66		**15.82 +/- 6.95**	**5.18** $_{1,43.8}$	**0.03**
	GSH		**0.06 +/- 0.02**	**6.67** $_{1,7.32}$	**0.03**		3.31+/-6.72	0.24 $_{1,55.9}$	0.62
Random Effects			*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*
	Mother ID		*0.00*	*0.00*	*0.00*		*466.80*	*21.61*	*20.72*
	Litter ID		*0.02*	*0.13*	*86.42*		*0.00*	*0.00*	*0.00*
	Residual		*0.00*	*0.05*	*13.58*		*1786.40*	*42.27*	*79.28*

Table S5: Test of the shielding hypothesis: prediction 2.3.1. Linear mixed model exploring the link between oxidative stress markers and stage of reproduction, breeding status and their interaction. P-values highlighted in bold do not remain significant after correction using the false discovery rate procedure.

		Protein Carbonyl				**MDA**				**SOD**				**Glutathione**			
		N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value
Fixed effects	Intercept	313	0.06+/-0.25			357	0.11+/-0.23			295	0.11+/-0.24			313	-0.08+/-0.26		
	Stage of reproduction			1.32 $_{2,277}$	0.27			0.02 $_{2,325}$	0.98			2.22 $_{2,254}$	0.11			2.00 $_{2,269}$	0.14
	(Pregnancy)		0.49+/-0.29				-0.17+/-0.26				-0.29+/-0.27				-0.09+/-0.28		
	(Lactation)		-0.14+/-0.30				-0.35+/-0.29				-0.29+/-0.28				0.40+/-0.31		
	Breeding status (Breeding)		0.09+/-0.25	0.56 $_{1,302}$	0.45		-0.02+/-0.23	1.69 $_{1,348}$	0.19		-0.11+/-0.24	0.11 $_{1,267}$	0.74		0.04+/-0.26	0.23 $_{1,307}$	0.63
	Breeding status x Stage of reproduction			**5.60 $_{2,274}$**	**<0.01**			0.89 $_{2,302}$	0.41			0.06 $_{2,249}$	0.94			0.70 $_{2,257}$	0.50
	(Breeding x Pregnancy)		-0.73+/-0.32				0.18+/-0.29				0.08+/-0.29				-0.03+/-0.31		
	(Breeding x Lactation)		0.25+/-0.33				0.41+/-0.31				0.10+/-0.31				-0.35+/-0.34		
Random effects			*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*
	Mother ID		*0.07*	*0.27*	*7.37*		*0.00*	*0.00*	*0.00*		*0.05*	*0.22*	*5.10*		*0.15*	*0.38*	*14.71*
	Litter ID		*0.22*	*0.47*	*23.16*		*0.36*	*0.60*	*33.64*		*0.34*	*0.58*	*34.69*		*0.19*	*0.44*	*18.63*
	Residual		*0.66*	*0.82*	*69.47*		*0.71*	*0.84*	*66.36*		*0.59*	*0.77*	*60.20*		*0.68*	*0.83*	*66.67*

Table S6: Test of the shielding hypothesis: prediction 2.3.2. Models exploring the link between maternal investment as well as offspring survival and oxidative stress markers during pregnancy, with the relevant covariates. P-values highlighted in bold do not remain significant after correction using the false discovery rate procedure.

		Pre-natal investment				**Offspring' body mass at emergence**				**Survival to emergence**				**Survival to 12 months**			
		N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	F-value $_{DF}$	P-value	N	Estimate +/- se	Chisq	P-value
Fixed effects	Intercept	21	0.39+/-0.05			50	190.4+/-69.72			26	0.42+/-0.6			56	-	-	-
	Offspring age at emergence		-	-	-		1.93+/-1.62	1.42 $_{1,35.3}$	0.24		-	-	-		-	-	-
	Number of foetuses		-	-	-		-	-	-		0.38+/-0.22	3.07 $_{1,19.9}$	0.09		-	-	-
	PC		0.01+/-0.04	0.14 $_{1,14}$	0.71		8.45+/-9.59	0.77 $_{1,39}$	0.38		-0.12+/-0.17	0.52 $_{1,19}$	0.48		**-0.75+/-0.31**	**6.01**	**0.01**
	MDA		0.03+/-0.03	0.83 $_{1,15.7}$	0.38		-9.93+/-9.35	1.13 $_{1,18.5}$	0.30		-0.18+/-0.17	1.12 $_{1,11.1}$	0.31		**0.75+/-0.3**	**6.39**	**0.01**
	SOD		-<0.01+/-0.03	<0.01 $_{1,15.2}$	0.99		11.52+/-8.83	1.7 $_{1,11.6}$	0.22		0.11+/-0.16	0.46 $_{1,6.5}$	0.52		-0.02+/-0.25	0.006	0.93
	GSH		0.05+/-0.02	4.12 $_{1,9.3}$	0.07		3.06+/-9.50	0.10 $_{1,16.7}$	0.75		0.20+/-0.15	1.6 $_{1,8.7}$	0.23		**0.46+/-0.21**	**4.99**	**0.02**
Random effects			*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*		*Variance*	*Std.Dev*	*%*
	Mother ID		*0.00*	*0.00*	*0.00*		*850.64*	*29.17*	*26.15*		*0.00*	*0.00*	*0.00*		-	-	-
	Litter ID		*0.02*	*0.14*	*77.06*		*20.35*	*4.51*	*0.63*		*0.03*	*0.16*	*5.45*		-	-	-
	Residual		*0.01*	*0.08*	*22.94*		*2381.67*	*48.80*	*73.22*		*0.52*	*0.72*	*94.55*		-	-	-

Does foetus size vary with treatment type or oxidative stress?

Methods

To determine whether foetus size differed between provisioned and control females, we ran a linear mixed-effects model with foetus size as the response variable and treatment type (provisioned or control) as the explanatory variable. Foetus age was also included in the model to control for the size difference in foetuses depending on how far during gestation they were measured, and mother ID and litter ID were included as random effects.

To determine whether foetus size differed between oxidative stress levels, we ran a linear mixed-effects model with foetus size as the response variable, and the different oxidative damage markers and antioxidant markers (MDA, PC, GSH and SOD) taken at the time of the foetus measures as the explanatory variables. Foetus age was also included in the model to control for the size difference in foetuses depending on how far during gestation they were measured, and mother ID and litter ID were included as random effects.

Results:

Unsurprisingly, in both models, foetus size significantly differs with foetus age, where larger foetuses are older (see table S7 and S8). However, table S7 reveals that foetus size did not significantly differ between provisioned and control females. Mother ID explained approximately 7% of the leftover variance in foetus size, while litter ID explained approximately 64%. Table S8 reveals

that foetus size did not vary with oxidative stress, except for GSH, where GSH significantly differs with foetus size. Mothers with higher GSH levels at the time the foetus measures were taken, carried smaller foetuses. Here, mother ID explained approximately 14% of the leftover variance in foetus size, while litter ID explained approximately 79%.

Table *S7*: Linear mixed model exploring the link between *foetus size* and provisioning treatment. *Significant p-values are emphasised in **bold.***

		Foetus size			
		N	Estimate +/- se	F-value $_{DF}$	P-value
	Intercept	84	0.78+/-0.73		
Fixed effects	Foetus age		**0.10+/-0.02**	**16.87** $_{1,25.9}$	**<0.01**
	Provisioning treatment *(Provisioned)*		-0.32+/-0.20	$2.54_{1,11.1}$	0.14
			Variance	*Standard deviation*	*% Variance explained by random effect*
Random effects	Mother ID		0.07	0.27	7.03
	Litter ID		*0.66*	*0.81*	*64.48*
	Residual		*0.29*	*0.54*	*28.49*

Table *S8*: Linear mixed model exploring the link between *foetus size* and *oxidative stress*. *Significant p-values are emphasised in* ***bold.***

		Foetus size			
		N	Estimate +/- se	F-value $_{DF}$	P-value
	Intercept	54	-3.13+/-5.00		
Fixed effects	Foetus age		**1.39+/-0.46**	**$9.24_{1,24.7}$**	**0.01**
	MDA		0.16+/-0.12	$2.00_{1,46.9}$	0.16
	PC		-62.77+/-44.43	$2.00_{1,32.0}$	0.17
	GSH		**-0.41+/-0.12**	**$11.18_{1,35.7}$**	**<0.01**
	SOD		-0.80+/-0.53	$2.25_{1,19.7}$	0.15
			Variance	*Standard deviation*	*% Variance explained by random effect*
Random effects	Mother ID		0.28	0.53	14.00%
	Litter ID		*1.59*	*1.26*	*79.11%*
	Residual		*0.14*	*0.37*	*6.90%*

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