Maternal disease, nutrition and social experience: consequences for the next generation

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Abstract

Natural selection should favour individuals who are able to adjust their life history strategy and resource allocation in response to changing environmental and social conditions. I examined the flexibility of reproductive resource allocation by female mice in response to manipulation of their nutritional, immunological and social environment. I considered the consequences of their investment decisions for the next generation. In five separate experiments I found that:

1. **Food restricted females skewed their offspring sex ratio in favour of sons.**
2. **Ambient disease cues caused females to produce more resistant and less aggressive sons.**
3. **Direct parasitic infection prior to pregnancy caused females to produce more resistant sons with an altered hormonal response to socialisation.**
4. **Dominant females were less likely to become pregnant, suckled litters less post-partum, and produced more aggressive offspring.**
5. **Despite a mate-choice preference for subordinates, females which were mated with dominant males had bigger litters.**

These responses by females to variation in their local environment are likely to have fitness consequences for their offspring, and as such would be acted upon by selection. Further work should seek to gain insight into the mechanisms underlying these responses, and determine the adaptive or non-adaptive nature of the flexibility observed.

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My family has given me endless love and encouragement throughout, I cannot thank them enough for their patience and support. Their unwavering belief that I could do this is the reason I am able to present this study.

The thesis is dedicated to the memory of my wonderful supervisor Chris Barnard, who always gave me the freedom to be creative yet the guidance to be productive. I miss our conversations, which so often strayed far beyond biology, so very much.

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For Chris

Chapter 1: General introduction

This thesis investigates how individuals vary reproductive investment in relation to changes in their environment and body condition. The impact of variation in investment by one generation on life history patterns in the following generation is then considered. In a laboratory setting, the nutritional, social or immunological environment of female mice (*Mus musculus*) during their first reproductive bout is experimentally manipulated. The effects of these challenges on female fertility, litter size and composition, and maternal behaviour are recorded, and the longer term consequences of the maternal environment for offspring development and quality are examined.

The thesis contains five experimental chapters. In each, the reproductive responses of female mice to an environmental change are examined. In Chapter 3, female mice are faced with a mild food restriction challenge. In Chapters 4 and 5, females are exposed to indirect and direct immunological challenges respectively. The effect on their reproductive output, as well as the behaviour and immunocompetence of their offspring as adults, is examined. Chapters 6 and 7 consider the effect of social status on reproductive decision making. Separate experiments explore the effect of maternal and paternal rank on reproductive behaviour in females, and on the attractiveness and dominance of adult offspring. As each of these experiments examines shifts in life history strategy as a response to environmental variation, I will begin by briefly introducing life history theory.

1.1 Life histories

The study of life histories, and our fascination with adaptation in general, is probably motivated by the simple observation that despite the relentless pressure of natural selection, organisms do not do all things perfectly (Zuk & Stoehr, 2002). Without constraint, we would expect evolution to lead to the emergence of a "Darwinian demon", an organism which matures instantly, produces an infinite number of high quality young and lives forever (Partridge & Harvey, 1988; Reznick *et al.*, 2000; Roff, 2002). We assume that no such demon exists because of the constraint of limited resources, a constraint frequently illustrated with a life history pie (Figure 1.1).

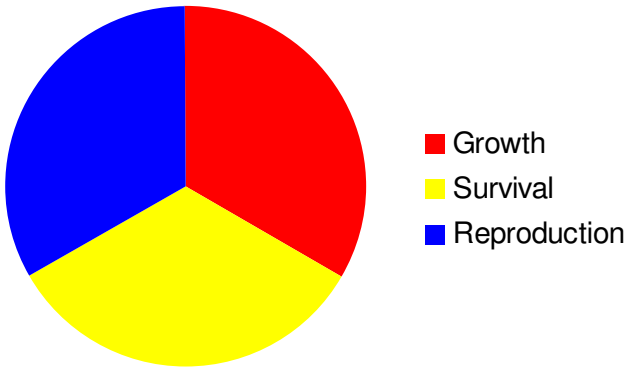


Figure 1.1: Limited resources constrain life histories. Here, the resources available are represented as a pie, with different slices being allocated to different functions.

We can imagine that whatever the resource – time, space, nutrients – each organism has only a fixed amount to allocate during its lifetime. The pie is divided into the slices of the organism's life history: the competing demands of reproduction, survival and growth (Price & Schluter, 1991; Shanley & Kirkwood, 2000). Because

the size of the pie is fixed, to increase the size of one slice means a necessary reduction in the size of another. In life history terms, a beneficial change in one trait leads to a detrimental change in another (Ketterson & Nolan, 1992; Stearns, 1989b; Tanaka & Suzuki, 1998; Thomas *et al.*, 2000).

The idea of limited resources leads to the hypothesis that adaptation is not free; it comes with costs (Zuk & Stoehr, 2002). Competitive allocation of resources into different components of life history underpins these costs (Reznick, 1992) and forms the basis for the idea of trade-offs. Trade-offs in life history traits result from common dependence on a limited resource, and are a fundamental concept in life history theory (Hardy & Kuh, 2002; Pease & Bull, 1988; Stearns, 1989b; Stevens *et al.*, 2000).

1.1.1 The cost of immune defence

Most studies of life history have been undertaken on large and visible plants and animals (Zuk & Stoehr, 2002). The organisms which shape these life histories are frequently much smaller (Altizer *et al.*, 2003; Barnard & Behnke, 2006; Barthelemy *et al.*, 2004; Clayton & Moore, 1997; Fitze *et al.*, 2004; Jolles *et al.*, 2008). Pathogens and parasites impose strong selective pressure on host life histories, forcing hosts to allocate resources to immune defence to the detriment of other life history components (Braude *et al.*, 1999; Hosken, 2001; Martin *et al.*, 2008; Sheldon & Verhulst, 1996; Tschirren & Richner, 2006). For example, immunologically challenged female blue tits (*Parus caeruleus*) and pied flycatchers (*Ficedula hypoleuca*) reduce the rate at which they feed offspring (Ilmonen *et al.*, 2000; Raberg

et al., 2000). Immunocompromised crickets (*Gryllus campestris*) sing less for mates (Jacot *et al.*, 2004) and bumble bees (*Bombus terrestris*) show reduced survival (Moret & Schmid-Hempel, 2000). Other examples illustrate how increased investment in reproduction or growth can cause a reduction in immune defence. Female odour causes male mice to invest in secondary sexual characters, leading them to subsequently lose more weight fighting an experimental infection (Zala *et al.*, 2008b). Zebra finches (*Taeniopygia guttata*), common eiders (*Somateria mollissima*), great tits (*Parus major*) and collared flycatchers (*Ficedula albicollis*) with larger broods all show reduced immunocompetence (Deerenberg *et al.*, 1997; Hanssen *et al.*, 2005; Nordling *et al.*, 1998; Richner *et al.*, 1995). Reproductive activity similarly reduces immunocompetence in fruit flies (*Drosophila melanogaster*) and damselflies (*Matrona basilaris*) (Mckean & Nunny, 2001; Siva-Jothy *et al.*, 1998).

The cost of immunity may arise because there is a finite resource, such as nutrients, which must be directed away from other fitness components to provide for resistance. This can be considered a resource cost (Zuk & Stoehr, 2002). Trade-offs may also come in the form of option costs – where functional or structural components of an organism must be dedicated to one thing or the other. For example, a single gene mutation may govern whether insects are resistant to a parasite, or a pesticide (Coustau *et al.*, 2000). Therefore relative resistance in populations will be governed by whichever threat is most potent. Similarly, haemocytes important in parasite resistance in adult *D. melanogaster* arise from the same precursor cells as the chewing muscle cells found in larval stages. One precursor cell cannot become

both mature cells, and so an option cost is paid for investment in the immune blood cells (Kraaijeveld & Godfray, 1997; Kraaijeveld *et al.*, 2001).

Finally, and uniquely to trade-offs in immunity, cost may come in the form of the risk of damaging immunopathology. Maximum immune response is rarely an appropriate reaction to attack, as damage to the hosts own cells and systems can occur though hypersensitivity and autoimmunity in vertebrates, and toxin production in invertebrates (McDade, 2005; Nappi & Christensen, 2005). Investment in immunity is consequently a fine balance, and resource input is strategic (Zuk *et al.*, 1996). When immune response is least effective (for example when the diversity of attacking organisms is broad and difficult to target), organisms should in fact invest less in defence, and more in other aspects of fitness (Jokela *et al.*, 2000).

1.1.2 Trade-offs within reproduction

So far, the discussed trade-offs in life history traits have largely been among, not within, the key components of fitness, as shown in Figure 1.1. In reality, differential allocation is much more complicated. Slices of the pie can be subdivided almost endlessly, as resource and option costs occur at different levels of allocation. Trade-offs also occur between different aspects of survival, growth and reproduction (Christians, 2000; Jordan & Snell, 2002; Martin Ii *et al.*, 2006; Roy & Kirchner, 2000) (Figure 1.2).

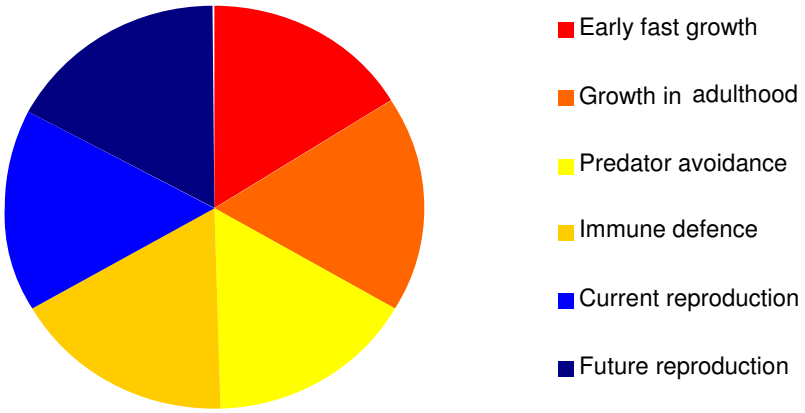


Figure 1.2: Slices of the life-history pie can be subdivided. Here, as in Figure 1.1, the available resources are represented as a pie with different slices being allocated to different functions.

Life history trade-offs have been considered a way of explaining why selection does not always favour having more offspring (Van Noordwilk & De Jong, 1986). Producing many offspring at a time may mean that fewer survive to breeding age (Van der Werf, 1992; Williams, 1966). This was first and most famously demonstrated by Lack (1947; 1964) who increased or decreased common swift (*Apus apus*) clutch sizes and found that either manipulation could lead to a decrease in the average number of offspring surviving to fledge. He defined the clutch size which would lead to the most offspring surviving as the "optimal clutch size", and explained this as the maximum number of offspring for which parents could provide adequate food. (Lack, 1947; Lack, 1954a; b; Lack, 1964)

Williams (1966) refined the Lack principle by considering iteroparous species, and subdivided reproductive effort into that which parents invest in the current

reproductive bout, and that which they save for future reproduction. This hypothesised trade-off has been supported by much empirical evidence (Gasparini *et al.*, 2006; Grüebler & Naef-Daenzer, 2008; Partridge & Harvey, 1985; Poizat *et al.*, 1999; Sheldon & Verhulst, 1996; Van der Werf, 1992; Verboven & Tinbergen, 2002). Within the current reproductive bout, trade-offs will also exist: offspring quality is often negatively correlated with offspring number (probably a resource cost limitation), and male offspring cannot be female offspring (an option cost limitation) (Christians, 2000; Jordan & Snell, 2002; Penn & Smith, 2007; Stearns, 1989b; Williams, 2001). Individuals should endeavour to invest in reproduction in a way that maximises the rate of return on their investment – the way which leaves the largest number of descendants (Williams, 1966).

1.1.3 Trade-offs are not always evident as negative correlations

Investigators generally expect trade-offs to be evident as negative correlations between traits, as in the examples mentioned above. However, in many cases no correlation, or even a positive correlation, is found between traits for which we would expect a trade-off (Laguerie *et al.*, 1991). These unexpected relationships can come about in several ways.

Firstly, as shown in Figure 1.2, trade-offs may occur at different levels of life history. For example, if searching for a trade-off between reproduction and survival, our view may be limited if we consider only current reproductive output and immunocompetence. A negative correlation may not be found because the trait

investigated is engaged in a trade-off with another immeasurable or unconsidered trait (Jordan & Snell, 2002; Kokko, 1998; Pease & Bull, 1988). As any given trait is probably competing with many others for limited resources, Laguerie *et al*. (1991) explain that trade-offs may only be obvious if they are the only two traits competing for a given resource, or if they are involved early in the metabolic pathway.

Alternatively, or additionally, non-negative relationships among life history traits may reflect differences in individual ability to acquire resources. For example, golden orb weavers (*Nephila clavipes*) use choline both as web material and for various internal functions (Higgins & Rankin, 1999). We might expect a negative correlation between the amount of choline found in the web and that found in the spider's body, because one use reduces choline availability for the other use. However, in natural populations there is a positive correlation because individual spiders vary greatly in their ability to acquire choline, masking the underlying trade-off (Higgins & Rankin, 1999; Roff, 2002). A helpful human analogy is suggested by Reznick *et al*. (2000). If we assume people have a fixed budget, we might expect that people spending more on a house will spend less on a car. In reality, there is a positive correlation between the values of houses and cars. This is obviously because human incomes vary, just as spiders are able to find different quantities of choline (Reznick *et al.*, 2000; Strassman & Gillespie, 2002; Van Noordwilk & De Jong, 1986).

Finally, underlying negative relationships can be masked by phenotypic flexibility. Individuals may adopt varying transient strategies during their lives, depending on

their own condition and that of their environment (Gluckman *et al.*, 2005a; Kawecki & Stearns, 1993; MacColl & Hatchwell, 2002; Roff, 2002; Stearns, 1989a). This makes it much more difficult to measure trait interactions, especially in natural populations with complex and ever-changing environmental conditions (Both *et al.*, 2000). Galápagos lava lizards (*Microlophus delanonis*) produce few small eggs in times of low rainfall, and many large eggs when rainfall is high (Jordan & Snell, 2002). The relationship between clutch size and egg mass is therefore positive if compared across seasons and across years. However, if compared within time periods, the expected negative correlation is found.

This thesis further explores the complexity of phenotypic plasticity in life history trade-offs. It examines how individuals change their reproductive investment in relation to changing environments and body condition, and the impact of this plasticity on life history patterns in the following generation. All experiments are conducted on laboratory strains of the house mouse, *Mus musculus.*

1.2 Study species - *Mus musculus*

1.2.1 Evolution, phylogeny and distribution

Rodents are the most abundant, diverse and widely distributed mammalian order, making up 44% of all mammal species (Honeycutt *et al.*, 2007; Jansa & Weksler, 2004; Wolff & Sherman, 2007). The order is made up of 33 extant or recently extinct families, but 89% of the 2,227 rodent species fall into one of five main families: Muridae, Cricitidae, Sciuridae, Echimyidae, and Nesomydae (Figure 1.3). Two thirds of rodent taxa are represented by the Muroidea (rats, mice, hamsters, voles, lemmings), which are found in abundance across all continents apart from Antarctica (Honeycutt *et al.*, 2007; Wilson & Reeder, 2005).

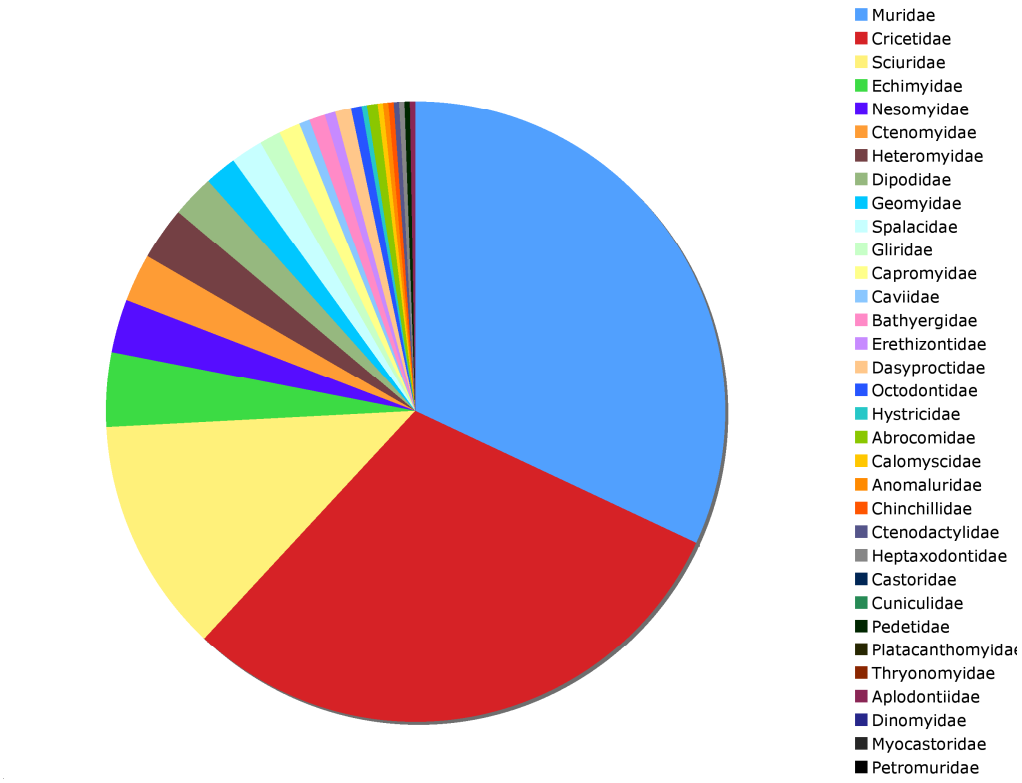


Figure 1.3: Distribution of extant and recently extinct rodent species across families, based on Wilson and Reeder (2005)

Rodents appear in the fossil record in the Paleocene, approximately 55-60 million years ago (mya) (Hartenberger, 1998; Reyes *et al.*, 2000), and appropriately calibrated molecular techniques support this date for the origin of the Rodentia (Adkins *et al.*, 2001; Bromhan *et al.*, 2000; Huchon *et al.*, 2002). The divergence of the murid genera (*Rattus* and *Mus*) is suggested by fossil data to be 12-14 mya (Jacobs & Pilbeam, 1980), whereas the molecular data suggests a figure closer to 24 mya (Adkins *et al.*, 2001; Honeycutt *et al.*, 2007; Huchon *et al.*, 2002). Phylogenetic relationships similarly vary depending on the whether morphological or molecular data are used (Honeycutt *et al.*, 2007), but some general consensus can be reached about the relationships between families (Figure 1.4) (Carleton & Musser, 2005).

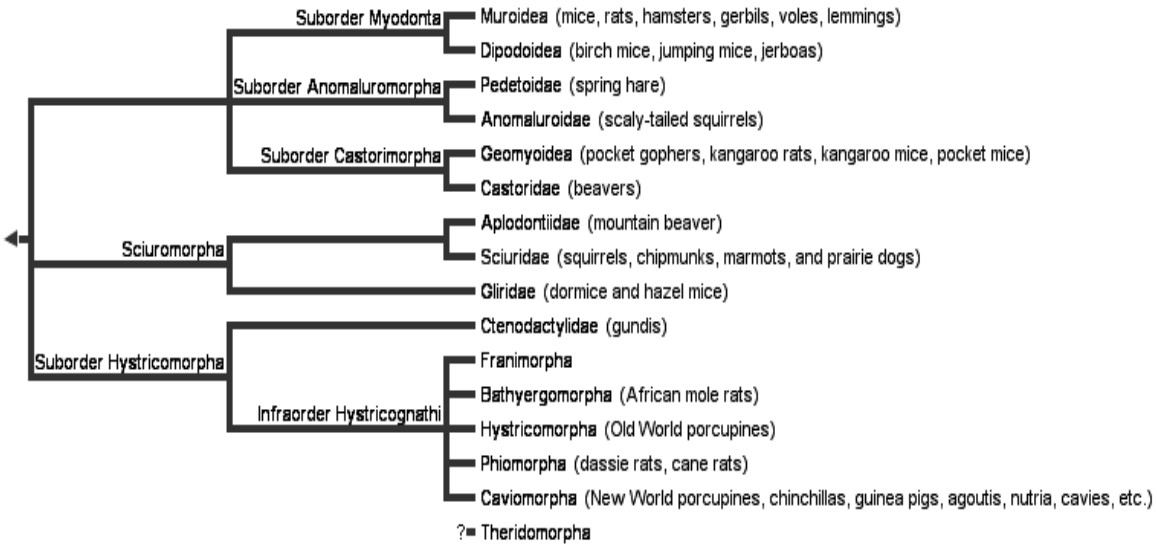


Figure 1.4: Phylogeny of rodent families, from Carleton and Musser (2005)

The first fossil representation of the *Mus* genus appears around 5 mya, and it underwent several radiations before the emergence of the house mouse, *Mus musculus,* 0.5 mya, with fossils found in Asia, Europe and Africa (Berdoy & Drickamer, 2007). *Mus musculus* is the accepted name for the now globally distributed species from which the laboratory mouse was derived, though some add the subspecies classification *Mus musculus domesticus* (Boursot *et al.*, 1993). Others consider *Mus domesticus* to be a separate species, though there is a hybrid zone in Europe (Berdoy & Drickamer, 2007; Bozikova *et al.*, 2005). For the purposes of this thesis the name *Mus musculus* will be used.

1.2.2 Biology and behaviour

Along with their much larger cousins, brown rats (*Rattus norvegicus*), common or domestic house mice are probably the most successful examples of commensal mammals living with human societies. Evidence suggests that mice began co-existing with humans around 10,000 years ago (Cucchi *et al.*, 2005). Berdoy and Drickamer (2007) helpfully define populations as urban or commensal if living in close association with us, or field or feral if living in more typically "wild" environments. Close living with humans has led to the damaging consequences of rodent-borne human disease and harvest loss, but the adaptability of rats and mice to human environments also makes them excellent laboratory animals (Hedges, 2002; Krebs *et al.*, 2007; Mackintosh, 1981; Sullivan, 2004). Mice were used in the laboratory as early as 1664, and following the generation of the first inbred strain 90 years ago, there are now more than 450 laboratory mouse strains described (Beck *et*

al., 2000). Mice now account for more than half of all vertebrates used in scientific research in Europe, and in the UK alone 2.2 million were used in 2007 (ASPA, 2007).

House mice show a variable social behaviour and spatial distribution depending on their surroundings. Commensal populations display male territoriality and polygyny, while field populations show more flexible overlapping ranges, promiscuity and multiple paternity (Berdoy & Drickamer, 2007). This probably reflects differences in population density between the two contexts, with commensal populations often containing over 10,000 mice/ha compared to only up to 100 mice/ha in field populations (Drickamer & Gillie, 1998). Mice are largely nocturnal, but will change foraging behaviour and daily rhythms depending on season, climate, and predation pressure (Berdoy & Drickamer, 2007; Brown *et al.*, 2001). As a largely nocturnal species, olfaction plays a key role in social and familial interactions, predator and parasite avoidance, navigation and foraging (Berdoy *et al.*, 2000; Brennan & Kendrick, 2006; Gelperin, 2008; Kavaliers *et al.*, 2005b; Keller *et al.*, 2006; Koyama, 2004; Penn *et al.*, 1998; Yamazaki *et al.*, 2000). The mouse genome was the first non-human mammal genome to be fully sequenced, and is 2.6 billion base pairs in size (Berdoy & Drickamer, 2007; Mitchell *et al.*, 1998; Rouquier *et al.*, 2000). There are approximately 1,510 genes dedicated to olfactory reception (Berdoy & Drickamer, 2007; Rouquier *et al.*, 2000).

In natural conditions the house mouse weighs 15-23g, though laboratory strains are often larger (Berdoy & Drickamer, 2007). Mice have a heart rate of 310-840 beats

per minute, and live up to 18 months in the wild, or 36 months in captivity. They reach sexual maturity at 55-80 days of age, and have a gestation period of 19-21 days. Litters size is between two and seven pups in field conditions (Berdoy & Drickamer, 2007), or up to 21 in the laboratory (personal observation). Female mice often co-operatively rear litters in communal nests, and prefer to nest with close relatives (Manning *et al.*, 1992).

Mice are an excellent study organism for the investigation of life history strategies and reproductive behaviour. Because of their small size, short generation times, and large litters, it is easy to obtain large sample sizes (Sikes, 2007). The ease with which mice can be kept in captivity, and their willingness to breed in the laboratory, also makes it possible to construct controlled experiments at relatively low cost (Beck *et al.*, 2000; Berdoy & Drickamer, 2007). The adaptability of mice to their social, physical, nutritional and immunological surroundings makes them a particularly ideal species given my interest in the interaction between environment and life history strategies.

Chapter 2: General Methods

This thesis contains five separate experimental chapters, each of which has a specific methods section detailing the experiment chronologically and describing new techniques used. The following general methods chapter describes materials and techniques which apply to all experimental chapters, unless otherwise stated.

2.1 Mouse strains

Inbreeding of mice leads to genetic homogeneity in the population, which is known to reduce fitness and impair individual recognition (Beynon & Hurst, 2004; Carroll *et al.*, 2004; Nevison *et al.*, 2000). This in turn impacts upon scent marking behaviour, mate choice and the maintenance of social dominance (Hurst *et al.*, 2001; Lacey & Hurst, 2005; Nevison *et al.*, 2003; Van Loo *et al.*, 2003), all of which are important in my experiments. In addition, there are related animal welfare consequences of inbreeding: the complexity of the social environmental is diminished, subordinate male mice are known to withhold urine, leading to renal pathology, and dominance instability can cause sudden extremely violent attacks (Jemiolo *et al.*, 1992; Nevison *et al.*, 2003; Van Loo *et al.*, 2003; Würbel, 2002). Given all of these considerations, I chose to use outbred mouse strains in all experiments.

Outbred CD1 mice are used in a range of behavioural and biomedical studies, and are known to form dominance hierarchies and breed well in captivity (Eveleigh *et al.*, 1983; Fitchett *et al.*, 2005; Nevison *et al.*, 1999). Therefore I used this strain,

supplied by Charles River UK, in all non-immunological experiments (Chapters 3, 6 and 7). For experiments with an immunological element (Chapters 4 and 5), I used outbred BKW mice from B & K Universal Ltd, Hull, UK, as the BKW-*Babesia microti* host-parasite system is well established in the laboratories at the University of Nottingham (Barnard *et al.*, 1997a; b; Barnard *et al.*, 1993; Barnard *et al.*, 1996a; Barnard *et al.*, 2005). BKW mice also readily form dominance dyads and breed well in captivity (Barnard *et al.*, 1998; Fitchett *et al.*, 2005).

2.2 Housing

I kept subjects in standard polypropylene cages (48 x 15 x 13cm: model M3, North Kent Plastics, UK). In the experiments in Chapters 4 and 7, larger divided cages were used for some phases of the experiments. All cages contained wood shavings as a floor substrate, a cotton nestlet for bedding material and a cardboard tube. Subjects had *ad libitum* access to standard laboratory rodent food pellets and water at all times except during food restriction periods, when only food was removed (Chapter 3). Room temperature was maintained between 20 and 22°C and humidity between 45% and 55%. All animals were maintained under a 12h:12h reversed light:dark cycle with lights on at 20.00h, and illuminated by a dim red light during the dark cycle to facilitate observations.

2.3 Behavioural observations

In all experiments maternal and pup behaviour was recorded between birth and weaning. Mice were marked using black eyelash dye (Colorsport, Brodie and Stone PLC, London, UK) to identify individuals or pup gender. Table 2.1 defines the pre-

weaning behaviours recorded (based on Barnard *et al.,* 1998). In Chapters 4 to 7, social dominance behaviour was also measured in social pairing phases. Table 2.2 defines social dominance behaviours recorded (based on Barnard *et al*. 1997 and Mackintosh, 1981).

Table 2.1: Behaviours and states recorded in pre-weaning phase.

States	
Hidden	Individuals are obscured from sight by bedding material, therefore activity can not be recorded
Contact (dam only)	Any part of the dam's body, other than the tail, in touch with at least one pup
Behaviours	
Suckling	Pups attached to teats of dam
Nest-building (dam only)	Dam uses teeth and paws to bring nesting material to the nest, or manipulates already incorporated material.
Feeding	Consumes provided food (suckling not included)
Resting	Asleep or motionless, usually in the nest
Grooming	Grooms own body parts
Allogrooming pup	Grooms or licks pups or another pup
Carrying pup (dam only)	Transports pup in mouth
Active	All other active behaviour e.g. moves around cage, consumes water, climbs bars

Table 2.2: Dominance behaviours recorded in focal mouse (A) towards cage-mate (B).

Behaviours	
Attack	A moves to bite or bites B, usually eliciting defensive posture and squeak in recipient
Defensive behaviours	Composite category combining flees, freezes, squeaks and defensively lifts forepaws upwards or to the side
Follow	A follows close behind B as B moves around the cage
Chase	A follows close behind B at speed causing flight
Aggressive allogroom	A roughly mouths and licks the fur of B, usually eliciting defensive posture and squeak in recipient
Bat	A pushes B out of the way with face or forepaws, often at resource such as food
Push down	A places both forepaws on top of B and forces B down, often causing displacement from resource such as food
Mount	A places forepaws over back of B, as if mounting for copulation
Social investigation	A sniffs or noses the body of B

2.4 Coat condition

In addition to behavioural cues, coat condition was scored on a scale of one to ten at various points in the different experiments. Scoring was done blind, based on anonymised photographs taken of dams or sires which were examined in a random order. Scoring was scaled based on extreme individuals at each end of the spectrum, as shown in Figure 2.1.

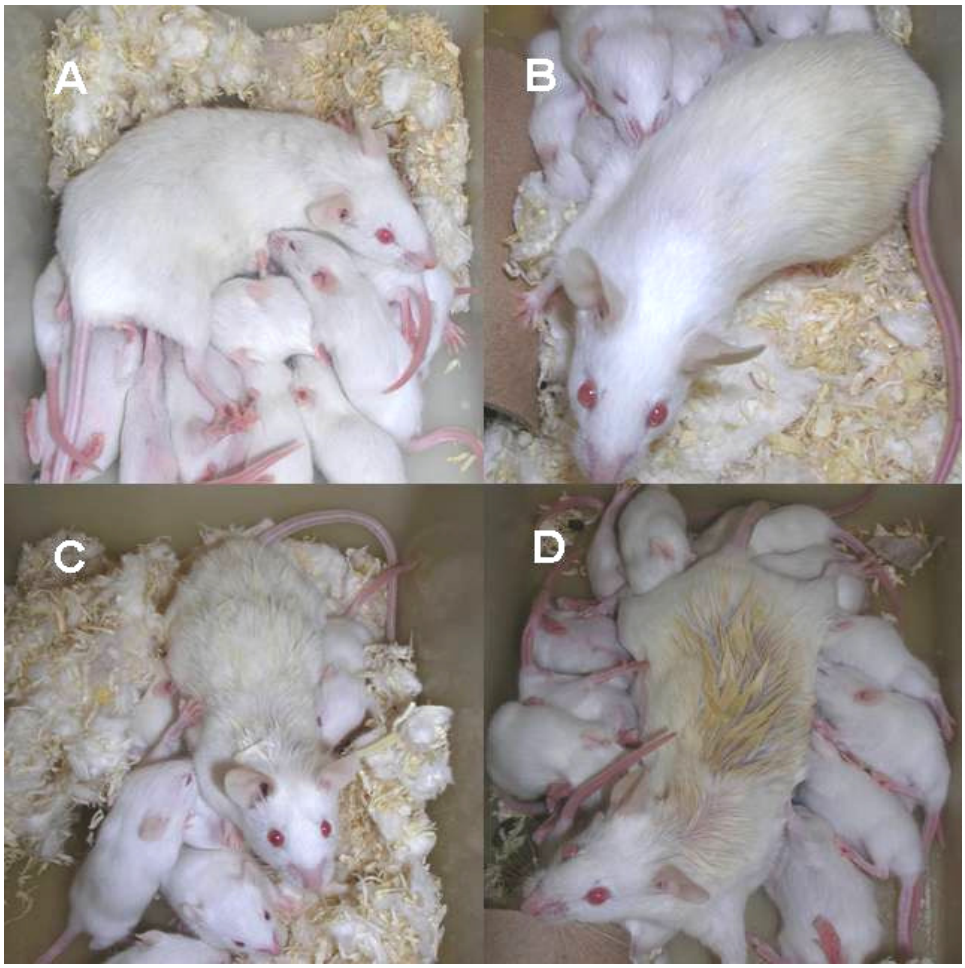


Figure 2.1: Coat condition scoring scale. For example, Mouse A scored 10, B scored 7, C scored 3, and D scored 1 out of a possible 10.

2.5 Nest building

Nests were scored from anonymised photos taken at 8am two days after individuals had been cleaned out at various points during the experiments. Nests were scored using a system adapted from those of Broida and Svares (1982), Bult and Lynch (1997) and Bond et al. (2002). The following scores were allocated reflecting the thermoregulatory value of nests:

1 = No clear nest present.

2 = Material assembled in a clear nesting area, but nest flat or slightly saucer shaped.

3 = Bowl-shaped nest with built up sides up to the height of the dam.

4 = Fully enclosed base and sides, built to the height of the dam or higher with a base at a level higher than the rest of the cage floor.

5 = Dome shaped nest with only a small opening present.

2.6 Licensed procedures for inoculation and monitoring of infections

Chapters 4 and 5 involved infecting mice with the blood parasite *Babesia microti* and taking blood in order to monitor infection and hormone levels. Experiments were carried out under Home Office Project Licences 40/2762, 40/2621 and my Personal Licence 40/7902. Adverse effects did not exceed the mild banding (as defined by the Home Office (ASPA, 1989)), and welfare and care of animals was considered throughout all procedures.

The King's 1967 strain of *B. microti* was used throughout, and frozen stock was first passaged five times in BKW mice before being used on subject mice. This was done

because frozen stock occasionally fails to cause infection. When frozen stock does successfully cause infection, it leads to low level and unpredictable infection profiles (Barnard *et al.*, 1994). Infections (or control sham-infections) involved a single intra-peritoneal injection of 200µl Hanks' solution, containing the appropriate inoculant.

To monitor *B. microti* infection, drops of blood were taken from a peripheral tail vein. The tail was nicked and a single drop of blood transferred to a glass microscope slide every other day during the infection period. The drop was immediately smeared to give a monolayer of erythrocytes, then fixed and stained. For staining, fixed slides were placed in a solution of one part Giemsa stain to three parts Sorenson's buffer for 40 minutes, before rinsing in Sorenson's buffer and drying in air. Larger blood samples (50µl) were also collected from the tail in heparinised haematocrit tubes, for use in hormonal and antibody analysis. This was done on no more than three occasions per animal, and never twice within a two week period. These blood samples were assayed by ELISA for testosterone, corticosterone and total IgG (used as a bystander measure of immunocompetence (Barnard *et al.*, 1996a)) using kits or reagents supplied by IDS Ltd, Tyne and Wear, UK (testosterone); R & D Systems Europe Ltd, Abington, UK (corticosterone); and Universal Biologicals, Cambridge, UK (IgG). All plates were processed using Microplate Manager 5.2.

2.7 Autopsy

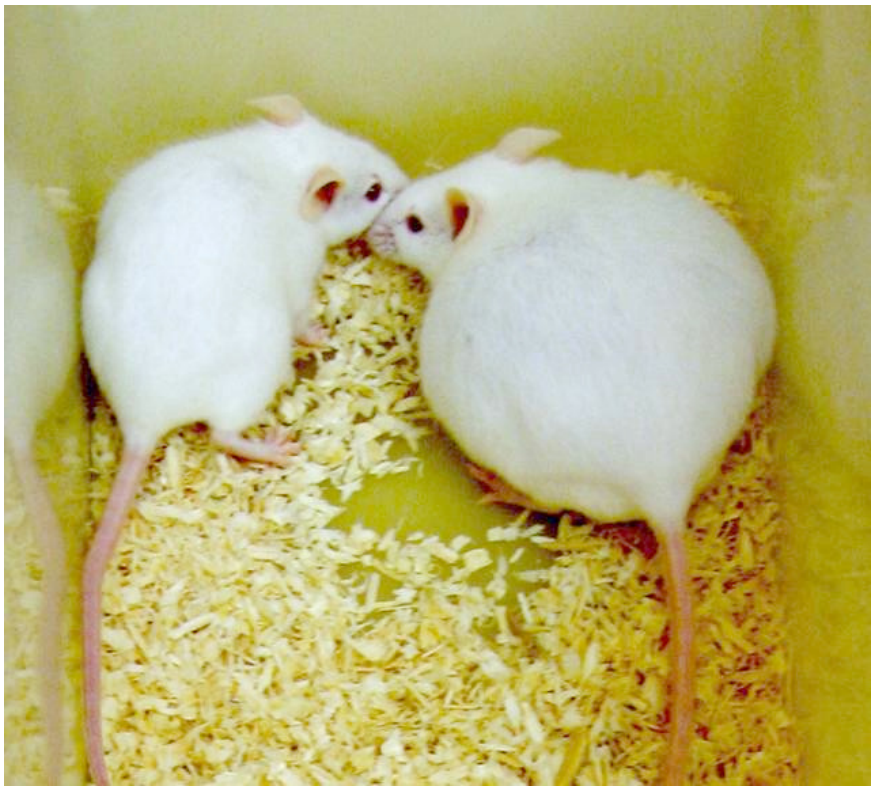
Autopsies were done on all adult mice following experiments because immunological, social and nutritional stress is known to have differing effects on

organ growth which can reflect pathological effects or adaptive investment (e.g. Barnard *et al*. 1997). The following organs were removed: thymus, heart, spleen, left and right kidneys, left and right adrenal glands (males and females), uterus (females), left and right testes, seminiferous tubules, and left and right preputial glands (males). All organs were weighed with the exception of the uteri, which were examined for implantation plugs in all females, including those that did not become obviously pregnant.

2.8 Statistical analysis

Analysis was carried out using SPSS 15 (SPSS Inc. Chicago, Illinois, USA). Alpha was set at 0.05 and all tests were two-tailed. Data were transformed to avoid violating the assumption of normally distributed residuals as required. Chi-squared tests were used to test for differences in frequencies among groups. General linear models (GLMs) were used to perform parametric analysis of covariance to assess differences in continuous variables among experimental groups, and the effects of important covariates (e.g. dam weight and litter size). Where individuals were monitored over time, repeated measures GLMs were used (e.g. when analysing infection profiles in Chapters 4 and 5). Pearson's correlations were used to test for associations among continuous variables. In several experiments, sample sizes varied among samples for some measures because not all females became pregnant, and because some litter sizes decreased during the maternal care period.

Chapter 3: Nutritional Challenge Experiment



3.1 Introduction

Access to nutrients varies due to environmental factors such as rainfall and predation risk, and due to changes in individual qualities such as age or social status (Appleby, 1980; Gosler, 1996; Holand *et al.*, 2006; Jordan & Snell, 2002; Verdolin, 2006). It is important for individuals to respond to this variability in a strategic way, investing in different life history components appropriately (Jordan & Snell, 2002). Nutrient availability, and its consequential effect on body condition, is known to affect reproductive output in a number of ways (Bronson, 1985; Fox *et al.*, 1997). Of particular interest to investigators has been the interaction between nutrients and

offspring sex ratio, which in many species appears to be skewed in a flexible and adaptive fashion (Rosenfeld & Roberts, 2004).

3.1.1 Sex ratio adjustment

Fisher (1930) proposed that stabilising selection would favour an equilibrium population sex ratio of 1.0. He argued that following any deviation from this equilibrium, producers of the rarer sex would be at a fitness advantage, and thus leave more descendants. His model was based on a number of assumptions which have since been explored and redefined (Maynard-Smith, 1980; Williams, 1979). Fisher's predictions have accordingly been adapted but his central idea of an equal sex ratio of 1.0 remains relevant, and population deviations from this equilibrium continue to stimulate interest and investigation (Charlat *et al.*, 2007; Dyson & Hurst, 2004; Hamilton, 1967; Meunier *et al.*, 2008; Wapstra *et al.*, 2007). Fisher's theory predicted the mean sex ratio at the population level, but not deviations which might occur in sub-groups or individuals within the population (Fisher, 1930; Hardy, 1997). Kolman (1960) considered this, and found that, provided that the population mean sex ratio remains equal, sub-group deviations will not be acted upon by stabilising selection. In fact, manipulation of the sex ratio by sub-groups or individuals might be highly adaptive (Kolman, 1960). This suggestion has led to several models predicting how individuals might skew sex ratios, or differentially invest in male or female offspring post-partum (Clark, 1978; Silk, 1983; Trivers & Willard, 1973).

Trivers and Willard (1973) predicted that polygynous individuals would differentially invest in male or female offspring depending upon their own condition. In this context, condition might be estimated by physical measures, such as body weight or fat reserves (Cameron & Linklater, 2000; Dittus, 1998) or by social rank. (Rank usually correlates with physical condition and may be determined by the outcomes of agonistic encounters and other behaviours (Alberts, 1994; Brown & Silk, 2002; Silk, 1988)). In polygynous species a male may mate with many females in a given breeding season, but a female will only mother the offspring of one male (Clutton-Brock & Isvaran, 2007). As there are necessarily equal numbers of male and female matings, if some males are to be highly successful and gain many mates, others must necessarily be less successful and gain few or none. In other words, there is high variance in male reproductive success. As females in polygynous species mate with only one male, they are not subject to the same intra-sexual competition, and have a low variance in mating success and reproductive success (Cameron & Linklater, 2000; Clutton-Brock *et al.*, 1981; Coltman *et al.*, 1999; Hiraiwa-Hasegawa, 1993; Penn & Smith, 2007). Given this gender difference in variance in reproductive success, Trivers and Willard (1973) predicted that high quality females might skew their sex ratio in favour of sons. This prediction rested on three assumptions:

1. Offspring condition at the end of the period of maternal investment correlates with maternal condition during maternal investment. Thus a high quality mother is better able to produce high quality offspring.
2. These differences in condition or quality persist into adulthood.

3. Adult males benefit more from good condition than females in terms of the effect on their reproductive success, as a result of the difference in variance in reproductive success between males and females.

A mother's behaviour will be selectively advantageous if it leaves her with the largest number of descendants. Selection can be expected to act upon not only those traits which affect her own fecundity, but also those affecting the fecundity of her offspring (Trivers & Willard, 1973). Therefore females in good condition were predicted to bias their maternal investment towards sons who would benefit most from their high quality. Meanwhile, females in poor condition were predicted to bias their investment in favour of daughters, who would be disadvantaged least by their mothers' poor condition. In other words, mothers will adopt a behavioural strategy which will generate the best long-term rate of return on their investment (Leimar, 1996; Lycett *et al.*, 1998).

3.1.2 Evidence of sex ratio adjustment

Since the publication of their seminal paper, the Trivers-Willard hypothesis has been tested in a variety of species both in the field and in the laboratory environment. In some cases the hypothesis has been supported. Several ungulates, in which female rank is usually correlated with body weight (Clutton-Brock *et al.*, 1981; Kojola, 1997), adhere to the predictions of the hypothesis particularly well (Hewison & Gaillard, 1999). High ranking female red deer (*Cervus elaphus*) produce more sons (60% male offspring) than low ranking females (46% male offspring) (Clutton-Brock

et al., 1984). High ranking arrui (*Ammotragus lervia*), and pronghorn sheep (*Antilocapra americana*) also produce more sons (Byers, 1997; Cassinello, 1996).

Primate populations have also been well investigated, and sometimes conform to the prediction of sex ratio correlating with female condition (e.g. chimpanzees (*Pan troglodytes*), rhesus macaques (*Macaca mulatta*)) (Bercovitch *et al.*, 2000; Boesch, 1997; Meikle *et al.*, 1984; Meikle & Vessey, 1988) and humans (*Homo sapiens*) (Almond & Edlund, 2007)). However, many primate populations show the opposite trend to that predicted by the Trivers-Willard hypothesis, with dominant or high quality females actually producing more daughters (e.g. bonnet macaques (*Macaca radiata*), yellow baboons (*Papio cynocephalus*) and captive rhesus macaques (Altmann, 1980; Gomendio, 1990; Silk, 1983; Simpson & Simpson, 1982). This unexpected direction of sex ratio skewing has been explained by the fact that primate societies are often complex, and maternal rank may be inherited by philopatric daughters but not dispersing sons. Similarly, daughters may compete with mothers for local resources, and therefore only good quality mothers with plentiful resources can "afford" to have daughters (Altmann, 1980; Clark, 1978; Hiraiwa-Hasegawa, 1993; Silk, 1983).

Rodents display polygynous mating systems with high variance in male reproductive success (Berdoy & Drickamer, 2007). While many species are extremely well described, the literature regarding facultative sex ratio skewing in rodents is still fairly sparse, and inconclusive (Rosenfeld & Roberts, 2004). Only a handful of studies show a positive relationship between female condition and sex ratio. Food

restriction prior to conception or a previous recent pregnancy has been found to skew maternal investment towards female offspring in mice and golden hamsters (*Mesocricetus auratus*) (Labov *et al.*, 1986; Rivers & Crawford, 1974), and female mice on high fat diets are found to have more sons (Rosenfeld *et al.*, 2003). However, many other studies have found no skewing in birth sex ratio due to maternal condition (Krackow, 1997; Sikes, 1996; 2007; Zamiri, 1978). In addition, the mechanism underlying reported examples of sex ratio adjustment in rodents remains unknown.

3.1.3 Nutrition and mammalian sex ratio adjustment

Despite the sustained interest in offspring sex ratio variation in mammals, the underlying physiological mechanisms have not yet been conclusively identified (Helle *et al.*, 2008). The availability of adequate nutrients has been suggested to play an important role in determining offspring sex ratio in some circumstances (Cameron, 2004; Green *et al.*, 2008). Variation in access to nutrients occurs both over time and among individuals in populations (Holand *et al.*, 2006; Sheldon & West, 2004). This heterogeneity creates variation in body condition which is expected to generate differential sex allocation in polygynous populations. When food is plentiful, females should produce more of the offspring which will benefit most from ample nutrients (Meikle & Westberg, 2001).

Elevated glucose in mice is known to lead to a male-biased sex ratio (Machado *et al.*, 2001) and this has been attributed to retarded growth of female conceptuses in the

presence of excess maternal glucose (Gutiérrez-Adán *et al.*, 2001; Helle *et al.*, 2008; Jiménez *et al.*, 2003; Larson *et al.*, 2001). Additionally, differential loss of male foetuses is the suggested mechanism in a case where low fat diets have led to smaller and female-biased litters (Rivers & Crawford, 1974). However, Rosenfeld *et al.* (2003) did not find any variation in litter size in response to dietary manipulation, but did find that females on a low fat diet produced more daughters than females on a very high fat diet, suggesting a mechanism at work other than differential foetal mortality (Rosenfeld *et al.*, 2003; Rosenfeld & Roberts, 2004). In their review, Rosenfeld and Roberts (2004) suggest various peri-conceptional mechanisms by which mammals, in which males are the heterogametic sex, might affect the offspring sex ratio in response to nutritional cues (Figure 3.1). It should be noted that more recent work has suggested selective fertilization (Figure 3.1B) is not possible in mammals (Zuccotti *et al.*, 2005).

3.1.4 A nutritional challenge experiment

Krackow (2002) stresses the importance of more research on the mechanisms underlying sex ratio adjustment, in order to prove that there is parental manipulation and hence adaptive sex ratio adjustment. The most recent findings suggest that body condition and diet around conception may hold the key to understanding how mammals adjust sex ratios (Cameron *et al.*, 2008; Cameron & Linklater, 2007). Therefore, in this experiment, I created a mild nutritional stress, and varied the timing of this stress in relation to conception among treatment groups. This was to attempt to gain insight into the sex-ratio skewing mechanisms that might be at work,

and specifically to exclude or provide support for some of the mechanisms seen in Figure 3.1.

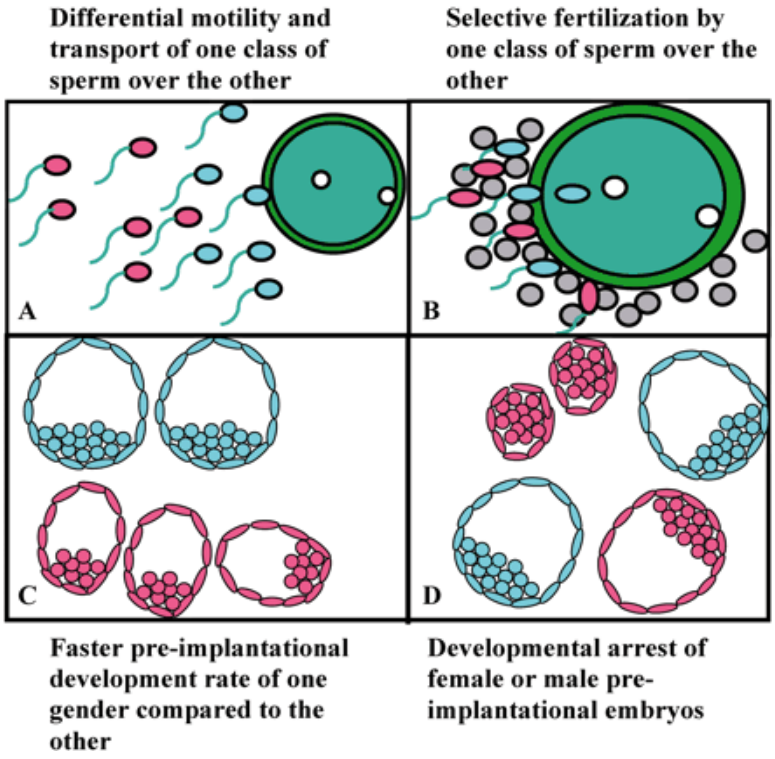


Figure 3.1: Proposed pre-implantational methods of sex-ratio skewing. From Rosenfeld and Roberts (2004).

Food was restricted for sixteen days either before mating, after mating, or for thirty-two days both before and after mating. For the reasons discussed above, I expected to see a sex ratio skew towards daughters in all food restricted groups. If only pre- or peri-conceptional sex-ratio skewing is possible in mice (e.g. Figure 3.1A and 3.1B), then I would not expect to see any sex ratio variation in the treatment group which was food restricted after mating. If however post-conceptional methods are used, such as foetal re-absorption or post-partum selective neglect, then all treatment groups might show a skew.

In addition, this experiment examined potential indicators of maternal investment strategy other than the primary sex ratio of litters. Many studies have focused on the sex ratio of offspring at birth, but relatively few have considered other estimates of maternal investment (Brown, 2001; Carranza, 2002). Post-partum maternal care varies greatly among individual mothers (Champagne *et al.*, 2007; Clark *et al.*, 1991; MacColl & Hatchwell, 2003; MacColl & Hatchwell, 2004), and may reflect a response to environmental cues as much as an individually consistent suite of behaviours. Therefore, in addition to examining offspring sex ratio and litter size, I recorded dam and offspring interactions between birth and weaning. I also used two other indicators of investment: nest building and coat condition.

Nest building behaviour varies among individual female mice, and is related to lifetime reproductive success (Bult & Lynch, 1997). Individual genetic variation, maternal experience, environmental temperature and hormonal fluctuations all affect nest building behaviour and the consequent thermoregulatory value of nests (Bond *et al.*, 2002; Broida & Svare, 1982; Bult & Lynch, 1997; Deacon, 2006; Voci & Carlson, 1973). I assessed the value of nests at various points in the experiment, both to examine whether or not nest building behaviour responds to food availability, and to see if there is an impact of nest quality on differential offspring survival. Finally, I scored female coat condition at the end of the period of maternal care as a bystander indicator of body condition at the end of the period of maternal care, and a potential indicator of the cost of investment in current, rather than future, reproduction (Bartolomucci *et al.*, 2004; Padgett *et al.*, 1998).

This experiment were therefore had three main objectives: Firstly, to provide a thorough experimental test of the basic idea of (potentially) adaptive sex-ratio adjustment in rodents, and thus to contribute to a currently inconsistent literature on the subject. Secondly, to contribute to the identification of the mechanism underlying sex-ratio skewing in mammals, by carefully manipulating the timing of nutritional stress. Finally, by examining differential maternal care post-partum, to test the hypothesis that maternal behaviour varies predictably in response to a pre-partum nutritional challenge.

3.2 Methods

3.2.1 Treatment groups

The subjects were 160 mice of the CD1 strain. This number comprised 80 subject dams (six weeks old) and 80 sire males (nine weeks old). Dams were randomly allocated to one of four treatment groups of 20 dams each (Figure 3.2). During food restriction periods (shown in red on Figure 3.2) food was removed from cages for three hours each day, during the active dark phase of the daily cycle.

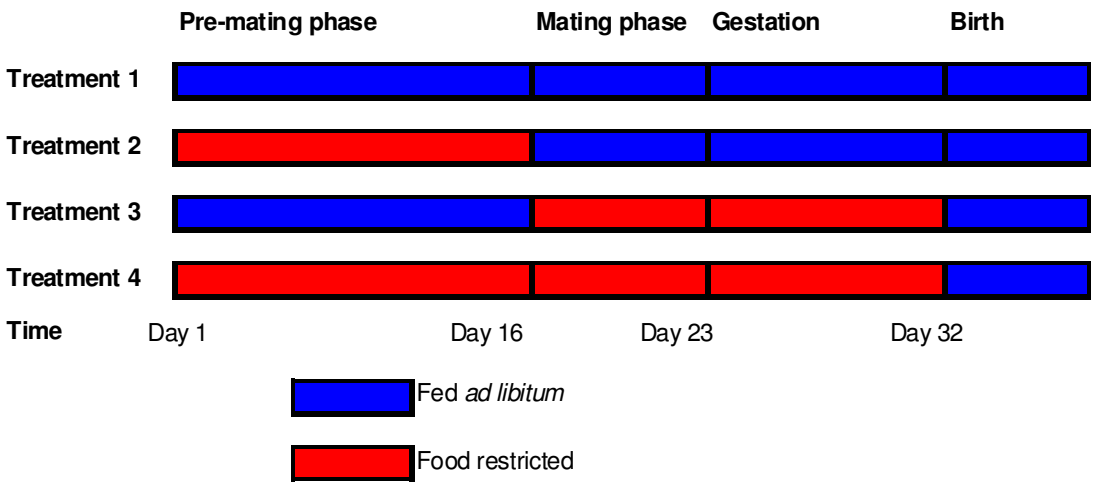


Figure 3.2: Treatment groups. This diagram shows the food restriction periods (in red) for each treatment group, when food was removed for three hours each day.

3.2.2 Mating phase

The mating phase began on day 16 of the experiment (see Figure 3.2), on which a single nine week old male mouse (previously socially housed in a group of four) was introduced to each female cage. Pairs were left for six days before removal of the male. Six days would be sufficient to allow impregnation of females (Berdoy &

Drickamer, 2007), but not expose sire males to an extended period of food restriction.

3.2.3 Pre-weaning phase

Four instantaneous scans per litter were taken each day between birth and weaning (21 days of age) to record dam and pup behaviours. Behaviours recorded are defined in Table 2.1 in the General Methods. In addition to behavioural cues, coat condition was scored on a scale of one to ten for each dam at weaning (see General Methods).

3.2.4 Nest building

Nests were scored from photos taken at 8am two days after females had been cleaned out (see General Methods). This was done at five points during the experiment:

1. Pre-mating, before the start of different treatments.
2. During treatment, prior to mating.
3. During pregnancy, treatment continuing.
4. Post-partum, pups approximately 1 week old.
5. Post-partum, pups approximately 2 weeks old.

3.2.5 Other measurements taken

In addition, dam, sire and pup weights were measured at regular intervals throughout the experiment. Litter sizes at birth, three, seven, fourteen and twenty-one days of age were recorded, and litters were sexed at fourteen days.

3.3 Results

3.3.1 Weight changes

There was no effect of treatment on dam weight prior to mating (see Table 3.1). However, dam weight during pregnancy was significantly affected by treatment (Figure 3.3: Repeated measures GLM: $F_{9,162}$ = 14.827, P = 0.034), with females which were food restricted during pregnancy (Treatments 3 and 4) showing a lag in weight gain, but no overall difference in weight gain over pregnancy (GLM: $F_{3,55}$= 1.305, P = 0.282). There was no effect of treatment on male weight over the mating period (Table 3.1), suggesting that males were not significantly affected by the food restriction regime during this time.

Table 3.1: There was no significant effect of treatment on the following measures.

	F	*Degrees of Freedom*	*P - value*
Dam weight change prior to pregnancy[a]	2.225	3, 55	0.095
Dam weight change during mating[a]	1.208	3, 55	0.315
Sire weight change over mating[a]	0.374	3, 76	0.772
Litter size at birth[a]	0.883	3, 55	0.456
Litter size at weaning[a]	1.129	3, 55	0.346
Nest scores[b]	0.734	9, 105	0.677

[a] General linear model
[b] Repeated measures general linear model

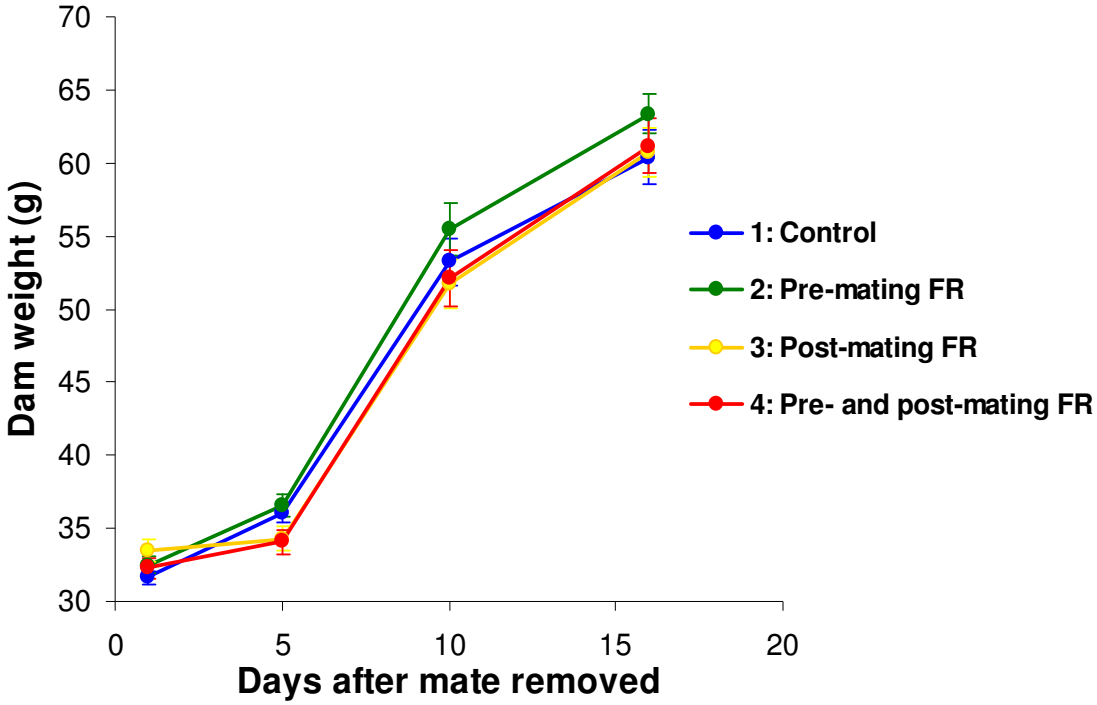


Figure 3.3: Dam weight changes over pregnancy. Dam weight (g) at various time points during pregnancy. Each line represents a different food restriction (FR) schedules, see Figure 3.2. Error bars represent ± 1 standard error.

3.3.2 Litter size and composition

There was no effect of treatment on litter size at birth or weaning (see Table 3.1), but treatment did significantly affect the number of pups lost during the period of maternal care (Figure 3.4: Repeated measures GLM: $F_{12,220} = 1.870$, $P = 0.039$). Dams which were food restricted during pregnancy, but not before, lost the highest number of pups post-partum.

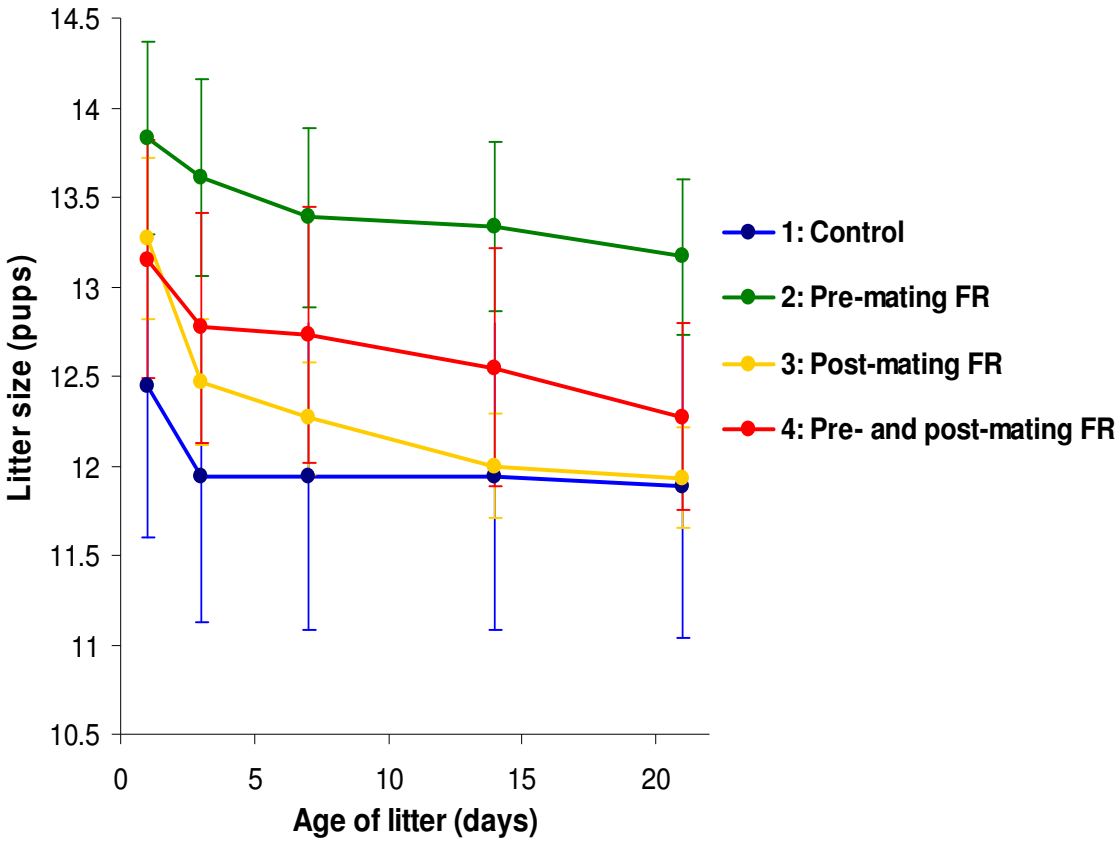


Figure 3.4: Changes in litter size during the period of maternal care. Each line represents a different food restriction (FR) schedules, see Figure 3.2. Error bars represent ± 1 standard error.

There was no significant effect of treatment on litter sex ratio, calculated as the percentage of male pups at weaning (GLM: $F_{3,55} = 1.404$, $P = 0.252$). However, examination of the data (Figure 3.5A) and the prior prediction that all food restriction groups would show a sex-ratio bias prompted me to pool the food restriction groups (Treatments 2, 3 and 4) and compare them with the control group. This comparison showed a significant effect of food restriction on sex ratio (Figure 3.5B: GLM: $F_{1,56} = 4.875$, $P = 0.031$), with food-restricted females producing more sons. Sex ratios of

the three food restriction groups were not significantly different ($F_{2,39}$ = 0.320, P = 0.728).

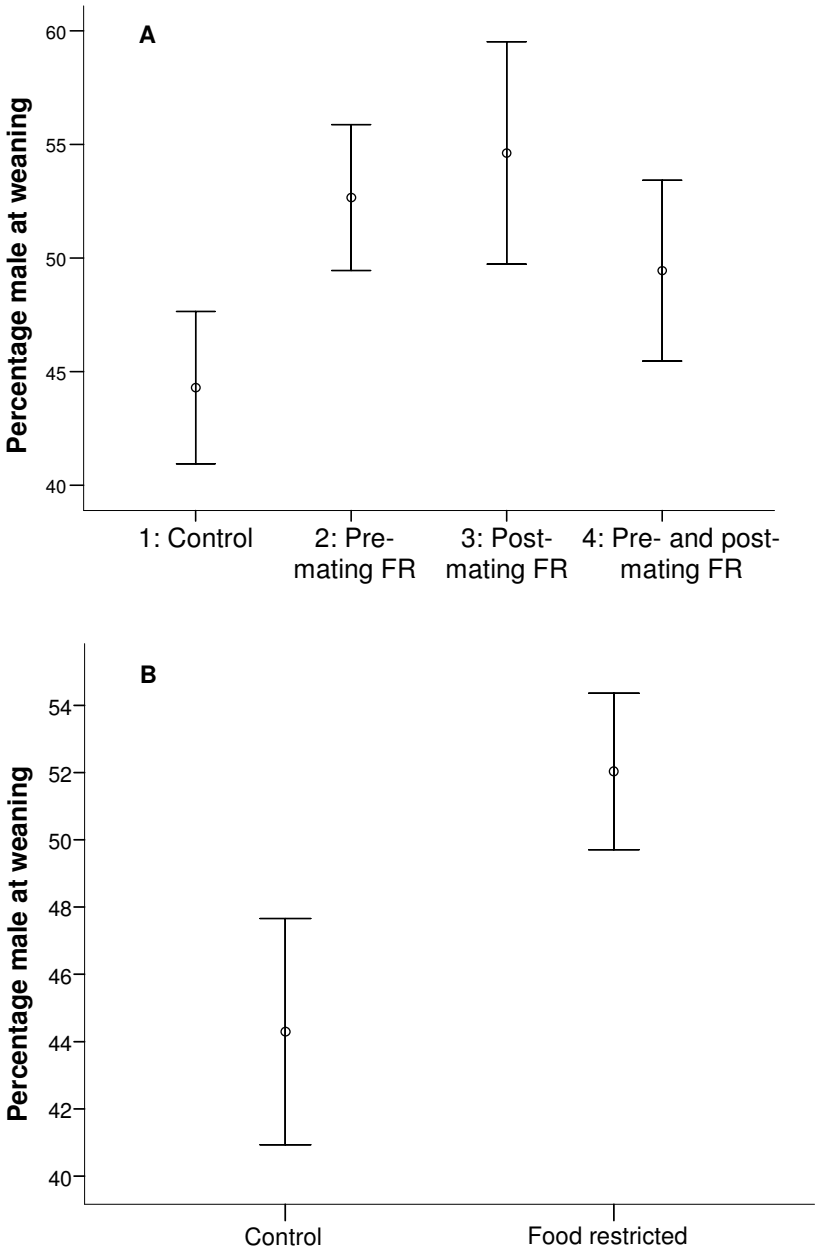


Figure 3.5: Sex ratio of litters. A: Treatments are shown separately, B: Food-restricted (FR) treatments are pooled. Sex ratio calculated as percentage male pups at weaning. Error bars represent ± 1 standard error.

There was a negative relationship between litter size at birth and average pup size at weaning (Linear regression: $R^2 = 0.362$, $F_{1,59} = 33.527$, $P < 0.0001$). This was not affected by treatment (Figure 3.6).

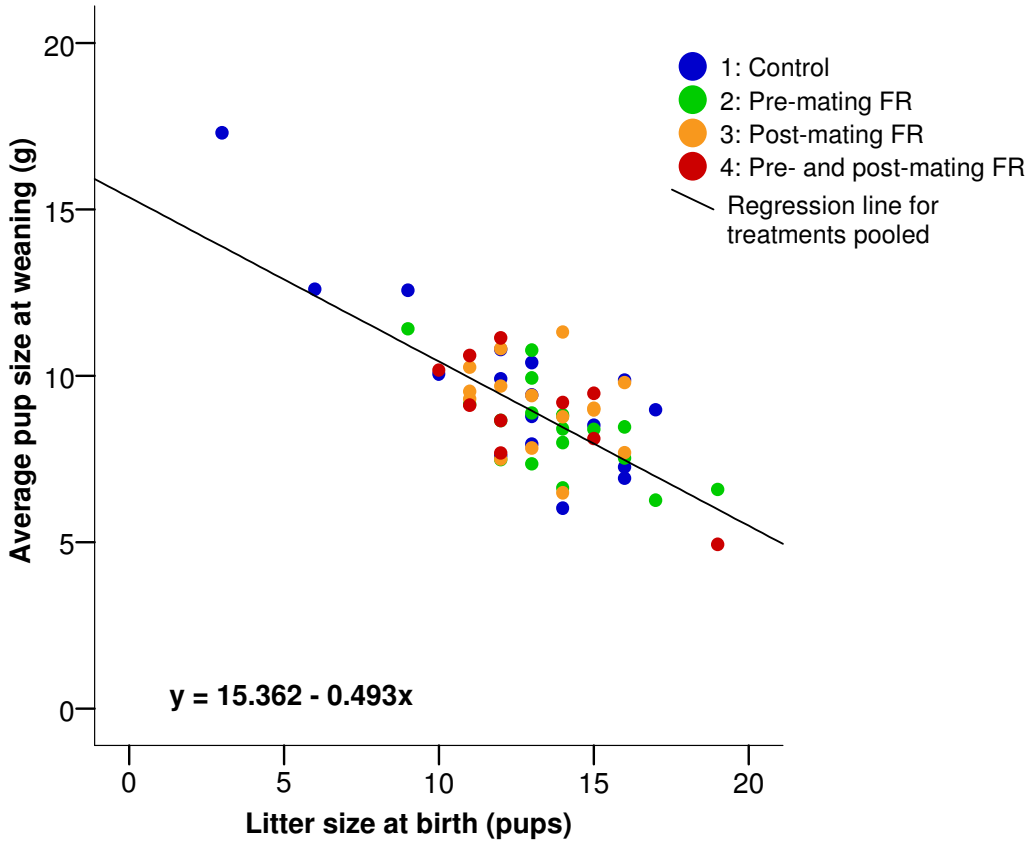


Figure 3.6: Negative relationship between litter size and pup size. FR = Food restriction.

3.3.3 Behavioural responses

The feeding rate of dams was significantly affected by treatment (Figure 3.7: GLM: $F_{3,55} = 3.505$, $P = 0.021$), dams which had been food restricted prior to mating spent significantly more time feeding during the pre-weaning phase. There was no other effect of treatment on dam behaviour pre-weaning (Table 3.2).

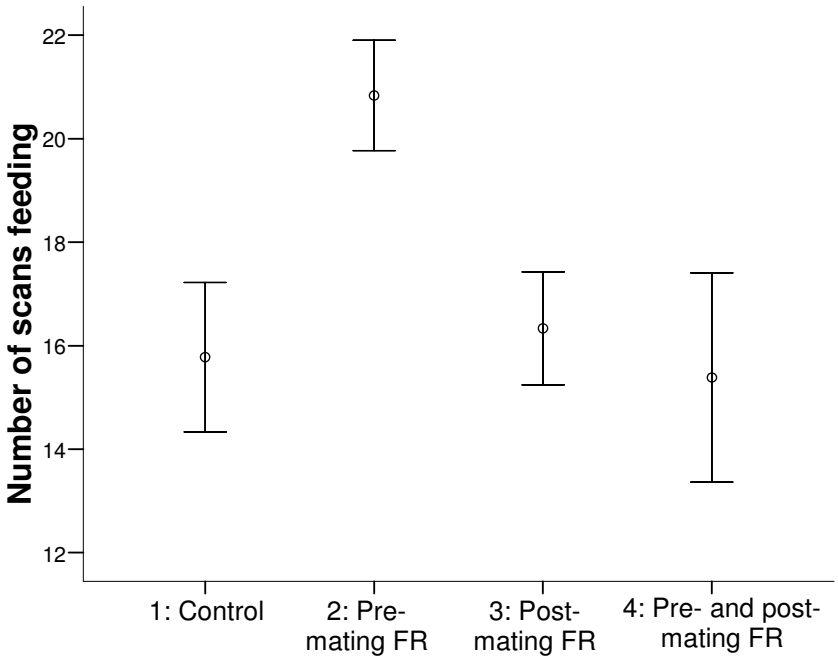


Figure 3.7: Dam feeding behaviour. The total number of scans in which the dam was observed feeding during the period of maternal care. Error bars represent ± 1 standard error.

Table 3.2: There was no significant effect of treatment on the following dam behaviours during the pre-weaning phase (all analysed with GLM).

	F	*Degrees of Freedom*	*P - value*
Suckling pups	0.813	3, 59	0.492
Resting	0.875	3, 59	0.459
Grooming pup	0.423	3, 59	0.737
Grooming self	0.472	3, 59	0.703
In contact with pups	0.353	3, 59	0.787
Active	1.912	3, 59	0.138
Carrying pup	1.881	3, 59	0.143
Nest building	0.519	3, 59	0.671

Coat condition was not affected by treatment ($F_{3,55}$ = 1.006, P = 0.397). Over the whole experiment, the quality of nest building was not affected by treatment (Table 3.1). However, there was a significant relationship between conception success and nesting behaviour. Dams which later became pregnant built nests prior to the

introduction of mates which had higher scores than those of females which did not later become pregnant (Figure 3.8: GLM: $F_{1,35} = 4.512$, $P = 0.041$).

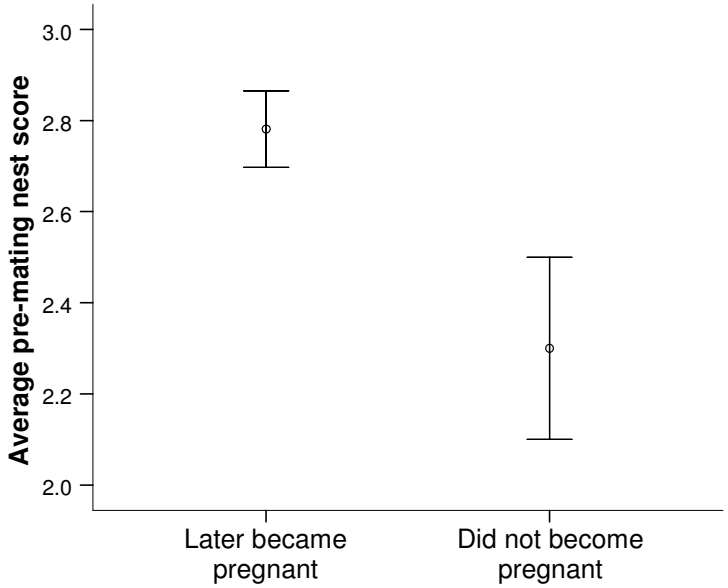


Figure 3.8: Dam nest scores. The average score of nest built before mating. Error bars represent ± 1 standard error.

3.4 Discussion

This experiment aimed to examine the effect of a nutritional challenge on reproductive output in female mice. In particular, it focused on the effect of food restriction on offspring sex ratios. In an attempt to gain mechanistic insight into facultative sex ratio adjustment, the timing of food restriction was varied relative to copulation among treatment groups. In stark contrast to the results found in previous studies, females from all food restriction schedules produced significantly male-biased sex ratios compared to control females, even if restriction ceased before conception. They produced litters which were on average 52% male, while control female litters contained 44% male pups.

Even though the restriction schedule was not severe enough to cause any weight changes in non-pregnant females or co-housed males, the results indicate that low level food restriction did impact upon reproductive output in females. Initially, it appears that females who had their food removed for three hours each day during pregnancy struggled to gain weight normally, but they adjusted to the restriction by the end of gestation (Figure 3.3). There was also an impact of food restriction on pup survival post-partum (Figure 3.4), with food restricted females losing more pups before weaning, particularly if they had not experienced food restriction prior to conception. These findings suggest that there is a negative impact of food restriction upon female condition. The Trivers-Willard hypothesis would therefore predict that food restricted females would bias the sex ratio of their litters towards the sex which would be disadvantaged least by their poor condition. As mice are polygynous, and

males need to be competitive to gain territories and therefore mates, I would expect food restricted females to produce more female-biased litters. Instead, I found that food restricted dams had significantly more male offspring than control females.

Frustratingly, there were only subtle differences among the food restriction treatments, limiting the mechanistic insight which can be gained from this experiment. Treatment 3 dams, which were food restricted only after the introduction of the male, varied most substantially from the control females in offspring sex ratio (Figure 3.5A). This weakly suggests that condition around or following conception plays an important role in sex ratio skewing, and that the mechanism at work might be dependent on sudden rather than long term changes in body condition. Meta-analysis of ungulate research supports a growing consensus that condition around conception is the crucial factor determining sex ratio (Sheldon & West, 2004). A larger study of mammalian data further supported this finding (Cameron, 2004). Most recently, compelling evidence suggests that a sudden change in condition around conception, rather than longer term condition, has the most dramatic effect on sex ratio (Cameron *et al.*, 2008). In this study, dams which had been subject to two weeks' food restriction prior to mating showed sex ratios closer to control levels than those suddenly restricted upon mating, but there was not a significant difference. Sex-ratio skewing as a response to changes in the immediate nutritional environment has been attributed to the differential responses of male and female conceptuses to circulating glucose (Cameron *et al.*, 2008; Larson *et al.*, 2001). Such a mechanism may have been at work in this study, but another key finding of this experiment points to a different possibility.

I found that treatment significantly affected pup loss post-partum. The treatment group with the highest pup loss also had the sex ratio which varied most from the control females (Figures 3.4 and 3.5A). This suggests that male-biased loss of pups post-partum may have been at least partly responsible for the skew seen. Unfortunately almost all of the pups lost died before they could be definitively sexed by non-invasive means, and dead pups were almost always eaten by their mother. Therefore it was not possible to determine the gender of pups lost prior to weaning. If differential pup loss was the mechanism by which sex ratios were modified, this might be due to selective maternal post-partum care, selective maternal cannibalism (Boesch, 1997; James, 2008), or differential vulnerability of male and female neonates to nutritional constraints (Stevenson *et al.*, 2000). Post-partum sex-ratio skewing mechanisms may be more likely in a highly polytocous species like *M. musculus*, where offspring are born altricially and some pup loss is normal (control females in this study lost an average of 5% of their offspring). In these species an additional trade-off occurs within each reproductive bout, with litter size being negatively correlated with pup size at weaning (Figure 3.6). It is suggested that this might add complexity to examination of sex-ratio skewing in rodents (Hardy, 1997; Servanty *et al.*, 2007).

Maternal behaviour was not found to be affected by treatment, though females which were food restricted before, but not during, pregnancy did spend significantly more time feeding in the maternal care period. Nest building behaviour was not found to be affected by feeding regime but interestingly did correlate positively with the

likelihood of becoming pregnant. Such a relationship has not previously been reported, but if fecundity is in some way correlated with nest building ability, this would increase the usefulness of this measure as an indicator of fitness (Bult & Lynch, 1997).

Of greatest significance in this study is the finding that food restricted dams produced more male offspring than controls, an opposite outcome to that predicted by the Trivers-Willard hypothesis. Females in good condition have previously been shown to produce more daughters in species where daughters are advantaged more than sons by maternal condition (Hewison *et al.*, 2005). For example, in species which display female philopatry (where sons but not daughters disperse) and daughters inherit maternal rank, dominant mothers produce fewer sons (Brown, 2001; Silk, 1983; Wild & West, 2007). Such social rank inheritance does not exist in mice, and in addition the evidence for alloparental care by earlier cohort daughters is limited (McGuire & Bemis, 2007). Perhaps of more relevance to this study is the possibility that high quality females will produce more daughters when there is a female-biased population sex ratio.

Following the removal of mates, female mice in this experiment were housed in a room full of the other experimental females – a local population sex ratio of 100% female. In a female biased social environment males are limited, and females become the sex with the higher variance, and lower mean, in reproductive success (and thus Trivers-Willard predictions might be reversed). Female lesser mouse lemurs (*Microcebus murinus*) vary their offspring sex ratio from 32% male (control group)

to 70% male when exposed to the urine of other females (Perret & Colas, 1997). Mice are known to similarly detect subtle cues about neighbouring individuals through social odours and might assess the social environment from these cues (Brennan & Zufall, 2006; Fernandes, 2000; Hurst *et al.*, 2001; Kavaliers *et al.*, 1998). In addition, females in this experiment experienced unnaturally high population densities, with over 40 in a small room. In most rodent species, sons disperse at a young age, and daughters are more likely to stay in the maternal territory and possibly assist in territorial defence (Berdoy & Drickamer, 2007; Krebs *et al.*, 2007). If able to assess the population density in the laboratory, pregnant females may have gauged that only high quality daughters would be competitive in such conditions, whereas sons would (under natural conditions) disperse and be less affected by local population conditions. I suggest that the female biased and densely populated local population in the laboratory may have provided cues (e.g. olfactory and auditory) that daughters would face more competition in this local environment, and would need to be of higher quality. If female are able to assess such cues and adjust reproductive investment accordingly, the patterns found in this experiment may have been adaptive. Examining the role of local population composition and density on reproductive output in rodents would be an interesting direction for future investigation.

There may also be non-adaptive explanations for the patterns of sex ratio skewing seen here. It is accepted that in sexually dimorphic species there are differences in growth rates between male and female foetuses and neonates, which may translate into differences in the vulnerability of each sex to changes in the maternal

environment (Forchhammer, 2000; Sikes, 2007). The ratios seen here may therefore have been a result of increased loss of males either pre- or post-partum. Food restricted dams in this study may also have been experiencing increased levels of stress. An unpredictable food supply may have led to physiological stress-responses, such as increased circulating corticosterone, and it may have been this, rather than direct nutritional changes, which led to the reproductive outcomes found (De Kloet *et al.*, 2005; Edwards & Burnham, 2001; Love *et al.*, 2005). Maternal stress might skew sex-ratios in unpredictable (and non-adaptive) directions, as it can have sexually differential impacts upon offspring behaviour and attractiveness in later life (Edwards & Burnham, 2001; Gluckman *et al.*, 2005b; Herrenkohl, 1979; Pratt & Lisk, 1989; Pratt *et al.*, 1989). Such long-term down-stream consequences might seriously impact upon optimal strategies, and in the following chapters I follow offspring after weaning and examine their competitive ability as adults.

Chapter 4: Indirect immune challenge experiment

The findings of this chapter have been published in the Proceedings of the Royal Society B: Biological Sciences. Please see Appendix.

4.1 Introduction

4.1.1 Social information

Animals gain remarkable levels of information from ambient social cues such as social odours (Brennan & Zufall, 2006). Cue recipients have been shown to be able to detect the species, sex, social rank, relatedness, fertility, familiarity, disease resistance, major histocompatibility complex class, major urinary protein profile and

individual identity of the cue donor (Barnard & Fitzsimons, 1988; Bean *et al.*, 1986; Bruce, 1959; Hurst *et al.*, 1994; Hurst *et al.*, 2001; Johnston, 1980; Jordan & Bruford, 1998; Lanyon *et al.*, 2007; Novotny *et al.*, 2007; Penn *et al.*, 2007; Roberts *et al.*, 2005; Stopková *et al.*, 2007; Thom & Hurst, 2004; Yamazaki *et al.*, 2000; Zala *et al.*, 2008b). This information can elicit behavioural and physiological responses (e.g. aggression (Hurst *et al.*, 1994) and growth (Cowley & Wise, 1972)) which have significant adaptive implications (Huck *et al.*, 1988b; Wyatt, 2003; Ziegler *et al.*, 2006).

Recently, researchers have considered the effect of temporary states such as stress or disease on social cues and the responses of receivers (Kavaliers *et al.*, 2003; Zala *et al.*, 2004). They have found that information from stressed or immunocompromised individuals can have dramatic and wide-ranging effects on the behaviour and physiology of neighbouring conspecifics. For example, female mice show preferences for the odours of unparasitised males in mate choice experiments (Ehman & Scott, 2002; Kavaliers *et al.*, 2005a; Kavaliers *et al.*, 2004; Kavaliers *et al.*, 2003; Penn *et al.*, 1998; Zala *et al.*, 2004), while healthy rats *(Rattus norvegicus)* mimic the organ growth and hormone production of immunologically challenged neighbours (Fernandes, 2000). In humans, body odour has long been used by physicians to diagnose diseases such as typhoid, where patients emit a baked bread odour, or yellow fever, were patients emit a meaty odour (Penn & Potts, 1998a). Thus it is known that many species of animals, including humans, are able to detect disease status in others, particularly in social odours (Kavaliers *et al.*, 2005b). It is

not known, however, if perception of this information benefits the receiver in any way, how it is used by individuals to make assessments about the risk of current disease in the immediate environment, and whether it is exploited subsequently to maximise reproductive success.

Information about social factors has already been shown to have dramatic effects on reproductive behaviour (Ebenspurger, 1998; Zala *et al.*, 2004). In gregarious species the social environment can be the most important factor determining the success of a reproductive attempt. Many mammals will terminate pregnancy if the social environment changes suddenly, in particular if the risk of infanticide increases because dominant males lose territories or harem owners are overthrown (e.g. lions *(Panthera leo)*, horses *(Equus quagga),* macaques *(Macaca mulatta)* and mice, (Beaton & deCatanzaro, 2005; Berger, 1983; Bertram, 1975; Bruce, 1959; Small, 1982)). In species with matrilineal dominance hierarchies, social pressure on subordinates can have similar effects (e.g. macaques and meerkats *(Suricata suricatta)* (Simpson & Simpson, 1982; Young *et al.*, 2006)). Social circumstances can also have more subtle effects. For example, cues about population composition can affect offspring sex ratio, gestation length, litter size, nesting behaviour, pregnancy success, maternal behaviour, menstrual cycle, menarche and menopause (Boesch, 1997; Cassinello & Gomendio, 1996; Colmenares & Gomendio, 1988; Drickamer, 1977; 1999; Gold *et al.*, 2001; Ma *et al.*, 1999; Manning *et al.*, 1992; Novotny *et al.*, 1999; Perret & Colas, 1997). Where these effects impact upon offspring phenotype, they are considered to be maternal effects.

4.1.2 Maternal effects

Maternal effects occur when factors in the maternal environment cause phenotypic changes in offspring (Agrawal, 2002; Clark & Galef, 1995; Clark *et al.*, 1993; MacColl & Hatchwell, 2003; Marshall & Uller, 2007; Mousseau & Fox, 1998; Rickard & Lummaa, 2007). These effects are independent of the genotypic contribution of the mother to the offspring or direct environmental effects on the offspring (with the exception of maternally controlled direct environmental effects, such as maternal care). More specifically, environmentally induced maternal effects, such as those being investigated by this study, are those which are initiated by an environmental stimulus in the maternal generation. In other words, they are the phenotypic product of the interaction between the parental genotype and that genotype's environment as expressed in the next generation (Lacey, 1998; Taborsky, 2006). These effects can be viewed as manifestations of transgenerational phenotypic plasticity (TPP) (Anway *et al.*, 2005; Anway & Skinner, 2006; Mousseau & Dingle, 1991).

Variation in offspring size at birth provides a useful example of a maternal effect. Mothers in a nutrient rich environment may increase the size of offspring at birth, which in turn can have major (non-genetic) effects on their size, survival and reproductive success as adults (Bernardo, 1996). Fox *et al.* (1997) found that female seed beetles (*Stator limbatus*) would vary the size of eggs laid depending on the host plant. On host plants for which large size was important for survival, large eggs were

laid. Where egg size did not impact upon fitness, smaller eggs were laid. As egg size determines emergent offspring size in this population, this work provides a neat example of TPP, with the effect of the laying environment in one generation impacting upon the phenotype of the next (Fox *et al.*, 1997). As fitness is substantially affected by egg size, this study also provides strong evidence for maternal effects being adaptive, and therefore illustrates how natural selection might act in a transgenerational way upon offspring phenotype. However, the adaptive nature of maternal effects is still being actively debated. Some suggest that perceived adaptive maternal effects may in fact simply be accidental products of gene-environment interactions with little impact on fitness (Groothuis *et al.*, 2005a; Groothuis *et al.*, 2005b; Marshall & Uller, 2007).

4.1.3 An indirect immune challenge experiment

Here, I twinned the growing fields of maternal effects and social information perception, to test the hypothesis that cues about the immune status of neighbours are exploited by female mice in adaptive reproductive decision making. Specifically, I tested the hypothesis that ambient information is used to gauge the threat of imminent disease to self and offspring, and that the capacity to resist infection in offspring in subsequent adult life is adjusted accordingly. I co-housed pregnant mice with conspecifics which had been either infected with a parasite, or given a control inoculation. The experimental set-up allowed multi-modal sources of information about the immune status of the stimulus neighbours, but no direct transmission of the

parasite. I then examined offspring social behaviour and response to disease as adults, as well various hormonal measures, to assess the impact of maternal experience on offspring life history.

I tested my hypothesis using BKW laboratory mice and the intraerythrocytic parasite *Babesia microti,* a well established host-parasite system (Barnard & Behnke, 2006; Barnard *et al.*, 2005; Smith *et al.*, 1996). *Babesia* species are tick-borne haemoprotozoan parasites that infect virtually all mammalian species, with significant economic consequences in domestic animals and with human health implications, particularly in the United States (Gorenflot *et al.*, 1998; Kim *et al.*, 2007; Kjemtrup & Conrad, 2000; Kuttler, 1985). In mice, *B. microti* induces high but transient parasitaemias, which are quickly cleared (clearance beginning approximately 10 days after infection) (Homer *et al.*, 2000). This makes *B. microti* an ideal parasitic tool to investigate the effects of disease in neighbours over a limited period, such as during pregnancy. In natural infections, antibodies can block *B. microti* sporozoites from invading erythrocytes shortly following infection. If this fails, NK cells and macrophages act to limit the extent of parasitaemia, by production of gamma-interferon (IFN-γ), tumour necrosis factor-alpha (TNF-α), nitrous oxide (NO) and reactive oxygen species (ROS) (Anstey *et al.*, 1996; Homer *et al.*, 2000) with suggested involvement of the recently discovered MetHb-pseudoperoxidase pathway (Bogdan, 2007; Jiang *et al.*, 2007). Ultimately, a resolution stage begins, with parasitaemia levels peaking and then rapidly declining due to the action of CD4+ T cells and IFN-γ (Homer *et al.*, 2000; Igarashi *et al.*, 1999). Following

primary infection, mice are protected against future infection by the action of CD4+ T cells and IFN-γ, with little or no requirement for B-cells or antibodies (Igarashi *et al.*, 1999).

The experiment mimics the heterogeneous environment created by variation in disease prevalence in the wild. Disease prevalence varies significantly among rodent populations. In fact, more variation in individual parasite load is attributable to population location than other intrinsic sources of variation (Bajer *et al.*, 2006; Behnke *et al.*, 2004). As a consequence, the current number of infectious animals can be used to predict the number of newly infected animals (i.e. risk of infection is dependent on the frequency of currently infected animals) (Hazel *et al.*, 2000). By placing pregnant dams in an environment with diseased conspecifics, I created an environmental effect with implications for offspring fitness, and tested the hypothesis that the threat of disease would induce a maternal effect measurable by phenotypic change in the offspring.

It is known that female mice can detect parasites in conspecifics, and can use this information to make decisions, such as mate choice (Willis & Poulin, 2000). This experiment was used to assess whether or not they also use this information to gauge the threat of disease in the environment, and whether they pass that information onto offspring in the form of maternal effects, thus demonstrating TPP. Ultimately, I considered whether such maternal effects were likely to be adaptive, impacting upon offspring disease resistance and other aspects of offspring life history.

4.2 Methods

Due to the complexity of the experiments in Chapters 4 and 5, a methods overview with an accompanying schematic figure is given in both chapters before the detailed methods.

Methods Overview

Figure 4.1 details the plan of experimental work. Pre-mated dams were housed on one side of a divided cage, separated from stimulus males by a clear perforated Perspex partition (Figure 4.2). Groups of stimulus males had either all been infected with the blood protozoan *B. microti*, or all subject to one of four control treatments. Dams were moved from divided cages to a new clean cage to give birth. At 11 days of age each litter was reduced to four males. Dams were removed from offspring at 24 days of age. At 50 days old approximately two-thirds of the offspring were re-housed with three novel males from the same treatment group. Continuous behavioural observations were carried out, recording aggressive behaviour in the adult offspring. At 70 days of age the adult offspring were singly housed and infected with *B. microti*. The time-course of the infection was monitored by blood smears until clearance. Blood was taken from dams at the start and end of the stimulus phase, and from offspring at weaning and before and after the social grouping and assayed for testosterone, corticosterone and total immunoglobulin G (IgG).

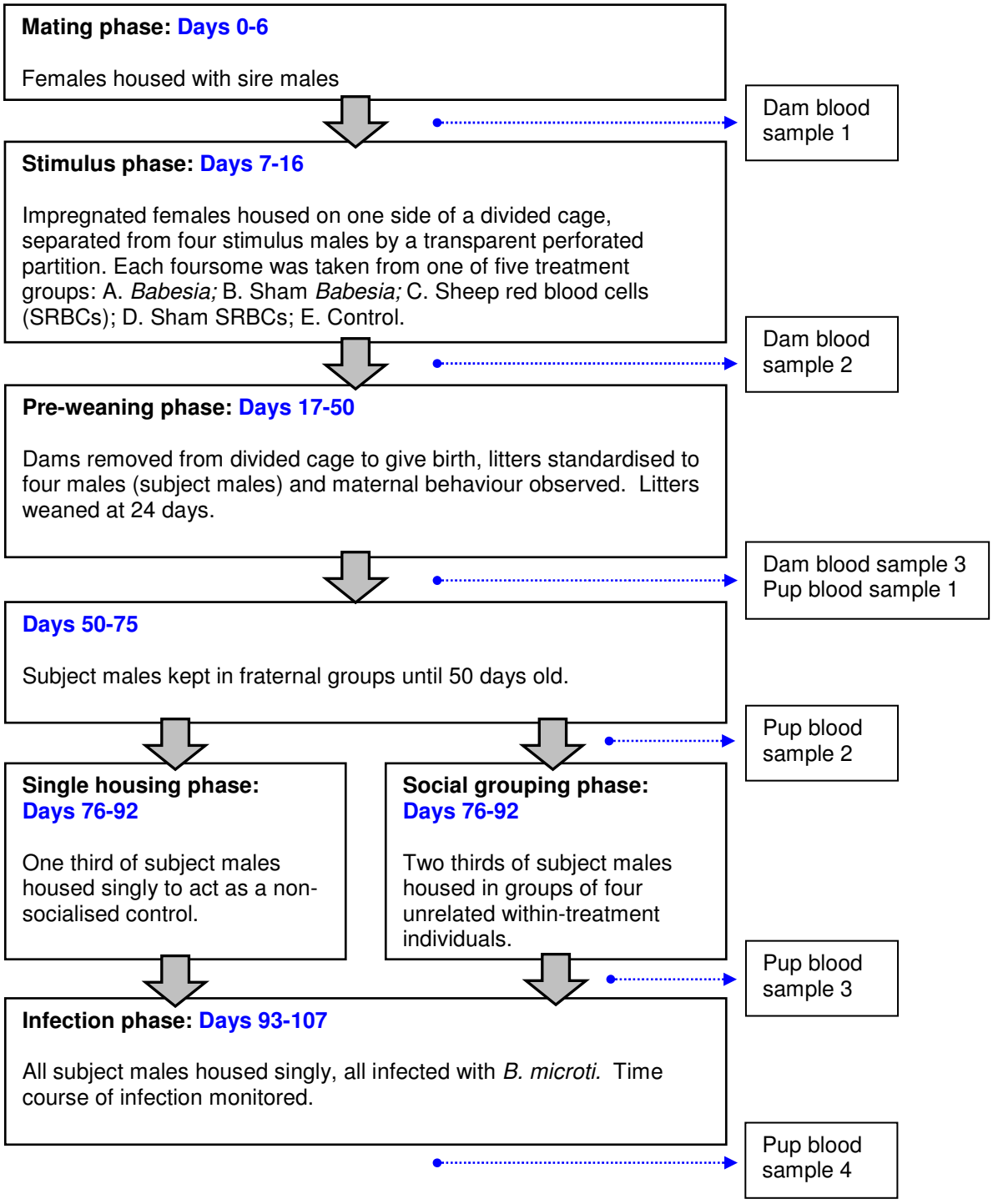


Figure 4.1: Plan of experimental work including timings of blood samples.

Figure 4.2: Dams housed on one side of divided cage, separated from stimulus males by a clear perforated partition.

Detailed methods

4.2.1 Mice and housing

A priori power tests were conducted to determine minimum sample sizes necessary to detect treatment effects of a magnitude that would be considered significant. This was done in order to minimise animal use. The subjects were 300 mice of the BKW strain. This number comprised 50 subject dams (seven weeks old); 200 stimulus males (three to five weeks old); and 50 sire males (nine weeks old). Subjects were kept in standard cages (see General Methods) except during the stimulus phase of the experiment when stimulus male groups and subject females each had one half of a

large divided cage (Figure 4.2, 28 x 45 x 13cm: model MB1, adapted specifically for this experiment).

4.2.2 Mating phase

Dams were introduced to the home cages of randomly allocated single-housed sire males, and kept in these pairs for six days. This method was used to minimise sire-dam aggression, and maximise the likelihood of impregnation (Koyama, 2004), whilst still leaving sufficient time for the stimulus phase.

4.2.3 Stimulus phase

Following the mating phase, each dam was separated from her sire and re-housed in a divided cage (Figure 4.2). The divided cage also housed four stimulus males, from which the dam was separated by a clear perforated Perspex partition. The divided cages were designed so that the dams could receive auditory, visual and olfactory information from their neighbours, but no direct contact. Stimulus males had been equally, randomly, divided into five treatment groups. Each group contained 40 subject males. The treatments were:

A. **Babesia treatment:** Infection with 5 x 10^7 red blood cells harbouring *B. microti.*
B. **Sham *Babesia*:** Sham infection of *B. microti,* comprising only the Hank's solution used to suspend erythrocytes in Treatment A.
C. **SRBC treatment:** Inoculation with 5 x 10^7 sheep red blood cells (SRBCs), which would elicit an immune response, but no pathology.

D. **Sham SRBC:** Sham inoculation of SRBCs, comprising only the Hank's solution used to suspend the SRBCs.

E. **Complete control:** No inoculation, but Treatment E males were handled, monitored and sampled in the same way as all other males.

Inoculations followed the protocols detailed in the General Methods. Immediately following inoculation, subject males were housed in within-treatment groups of four on one side of the divided cages. Subject dams were divided into one of five indirect exposure groups, with ten females per group. Direct transmission of *B. microti* to the dams was not possible as it is dependent on a tick vector (Randolph, 1991), which was absent, or direct exchange of blood, which was not possible. The absence of infection in dams was validated by testing of the dam blood at the end of this stimulus phase. Dams were kept in this stimulus phase for ten days. At the end of the stimulus phase, the dams were transferred to a new clean cage to give birth.

4.2.4 Pre-weaning phase

At eleven days of age, all pups were sexed and each litter reduced to four males (three males in the case of three litters that had only three males each). This was to enable manageable sample sizes for individual observations both pre-weaning and in subsequent phases. Male pups were chosen because previous work has shown interesting interactions between male sociality and immunocompetence (e.g. Barnard et al. 1994; 1997; 1998) and due to the limitations of the project licence. A total of 145 male pups were included in the following stages of the experiment, from 37

females. Thirteen dams did not become pregnant in the mating phase, but the likelihood of becoming pregnant was not affected by treatment (Chi-Squared test: $X^2 = 1.663$, $df = 1$, $P = 0.197$). Five instantaneous scans per day (totalling 80 scans per litter) were conducted until weaning, recording various dam and pup behaviours (detailed in General Methods Table 2.1). At 24 days of age all mothers were removed from litters, and pups (subject males) were left in their fraternal groups until 50 days old.

4.2.5 Social grouping and single housing phases

At 50 days of age all subject males were separated from their siblings. Approximately two-thirds of the subject males (88 mice) were re-housed with three novel males from the same treatment group, with whom they were allowed to establish dominance hierarchies. This was done to investigate the interaction between social rank and immunocompetence. Continuous behavioural observations totalling 175 minutes (10 or 15 minutes per day) per group were carried out. Various social interactions were recorded (detailed in General Methods Table 2.2), in order to determine social rank within these groups. The remaining third (57 mice) were housed singly to act as a non-socialised treatment. At the end of this phase all males were removed and housed singly in new clean cages.

4.2.6 Infection phase

At 70 days of age the adult offspring, all now housed singly, were injected with 5 x 10^7 red blood cells infected with *B. microti*. The time course of the infection was

closely monitored until clearance (for details of monitoring procedures, see General Methods).

4.2.7 Technical procedures

All inoculations, infections and sham manipulations involved a single intra-peritoneal injection of 200µl Hanks' solution, containing the appropriate inoculant. Stimulus treatment Group E received no injection. All monitoring, sampling and handling for stimulus treatments A and C were repeated accordingly in sham treatments B, D and E. Sham infection and inoculation involved the introduction of Hanks' solution only. All subject males (the adult offspring) were infected in a random order with 5 x 10^7 infected red blood cells of *B microti.* Infection was monitored by blood smears, and 50µl blood samples were taken from all subjects at various points in the experiment (see Figure 4.1) to monitor testosterone, corticosterone, and IgG. In a number of cases, limited serum volumes precluded reliable estimates for all three serum factors at all time points from certain individuals. As a consequence, sample sizes of some analyses vary. An additional blood sample was taken from all animals during autopsy. For details of blood sampling techniques and assays see General Methods.

4.2.8 Statistical analysis

All analyses were based on pooled control treatment groups B-E as no significant differences were found among these groups (see Table 4.2). Where appropriate, values were nested within dam (which was included as a random factor), or averaged

per dam to avoid within-litter pseudoreplication. Principal components analysis conducted on five correlated variables describing individual infection profiles generated a single principal component which captured the majority of the variation in the characteristics of the profiles (PC1). Individual scores for this component were then used in further analysis.

4.3 Results

4.3.1 Physiological effects on subject dams

All analyses were based on pooled control treatment groups B-E as no significant differences were found among these groups (Table 4.1). Pregnant dams housed opposite infected males (*Babesia* treatment) had blood serum levels of corticosterone during the stimulus phase that were twice the values recorded from dams housed opposite control males (Absolute corticosterone following stimulus phase: GLM: $F_{1,20} = 4.897$, $P = 0.039$; Change in corticosterone: Figure 4.3A, $F_{1,20} = 4.931$, $P = 0.038$). Also, dams from the *Babesia* treatment had kidneys that were 8% larger at autopsy than those of dams in control treatments (Figure 4.3B, GLM: $F_{1,34} = 9.391$, $P = 0.004$). There was no effect of treatment on dam IgG (Repeated measures GLM: $F_{2,33} = 0.819$, $P = 0.494$) or any other dam organ (Table 4.2).

Table 4.1: No differences were found among the four control treatments (see Methods) in the following physiological and behavioural measures.

	F	***Degrees of Freedom***	***P - value***
Change in dam corticosterone[a]	0.109	3, 12	0.953
Post exposure dam corticosterone[a]	0.096	3, 12	0.961
Dam paired kidney weight[a]	0.208	3, 24	0.890
Offspring aggression in novel groups[a]	0.844	3, 25	0.765
Infection profile[b]	1.480	15, 63	0.140
Infection profile PCA score[a]	0.652	3, 23	0.584

[a] GLM
[b] Repeated measures GLM of measurements taken across the experiment

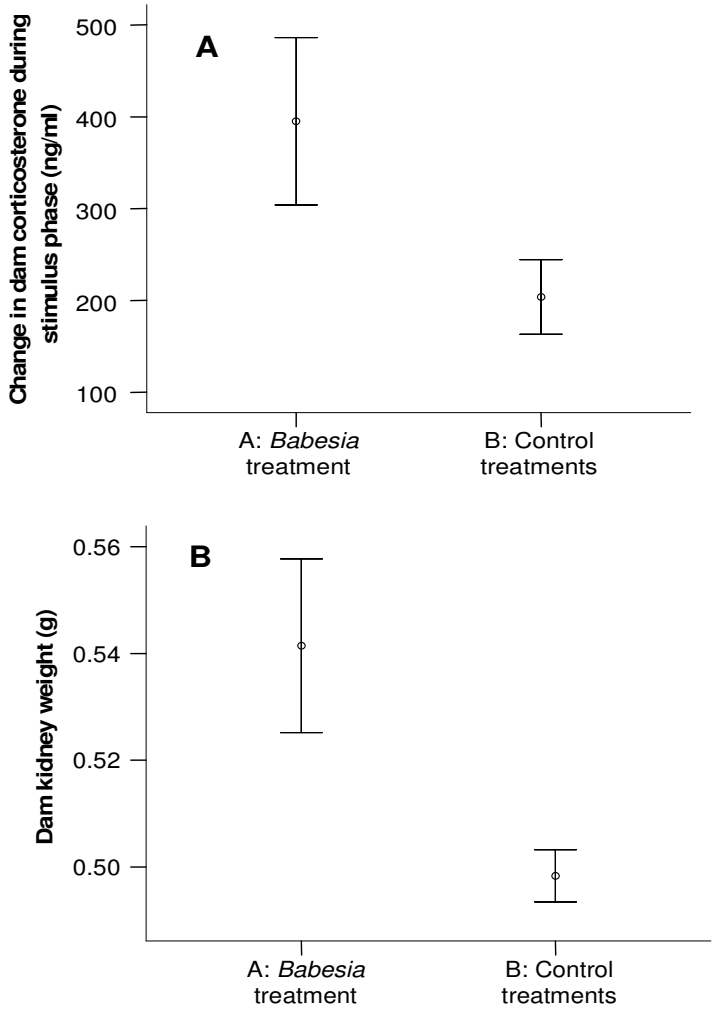


Figure 4.3: Treatment effects on dam physiology. Differences between dams that had shared partitioned cages with males infected with *B. microti* (A) and those that shared with control males (B) **A,** change in dam corticosterone across stimulus phase, and **B,** combined weight of both kidneys of dams at autopsy. Error bars represent ± 1 standard error (SE).

Table 4.2: No effect of treatment was found on various physiological and behavioural measures.

	F	*Degrees of Freedom*	*P - value*
Stimulus males active behaviour[a]	0.983	1, 35	0.328
Stimulus males resting behaviour[a]	0.669	1, 35	0.419
Stimulus males feeding behaviour[a]	1.882	1, 35	0.179
Dam spleen[a]	1.537	1, 34	0.224
Dam heart[a]	0.310	1, 34	0.582
Dam thymus[a]	2.708	1, 34	0.109
Dam paired adrenal glands[a]	0.840	1, 34	0.366
Litter size at birth[a]	0.612	1, 35	0.439
Litter sex ratio[a]	0.837	1, 35	0.367
Dam immunoglobulin[b]	0.819	2, 33	0.494
Offspring testosterone[b]	1.708	3, 33	0.184
Offspring corticosterone[b]	0.571	3, 33	0.638
Offspring immunoglobulin[b]	1.021	3, 33	0.397
Offspring aggression pre-weaning[a]	2.028	1, 35	0.163
Dam suckling behavior[a]	2.137	1, 35	0.153

[a] GLM
[b] Repeated measures GLM of measurements taken across the experiment

4.3.2 Effects on offspring behaviour and hormones

Offspring from *Babesia*-treatment dams were significantly less aggressive than those from dams in control treatments (Figure 4.4, GLM: $F_{1,42} = 5.508$, $P = 0.024$). While testosterone levels in offspring did predictably (Barnard *et al.*, 1994) correlate with aggression (Pearson's correlation: $n = 86$, $r = 0.295$, $P = 0.006$) experimental treatment was not found to have a significant effect on this hormone (Repeated measures GLM: $F_{3,33} = 1.708$, $P = 0.184$). There was no effect of treatment on offspring IgG (Repeated measures GLM: $F_{3,33} = 1.021$, $P = 0.397$).

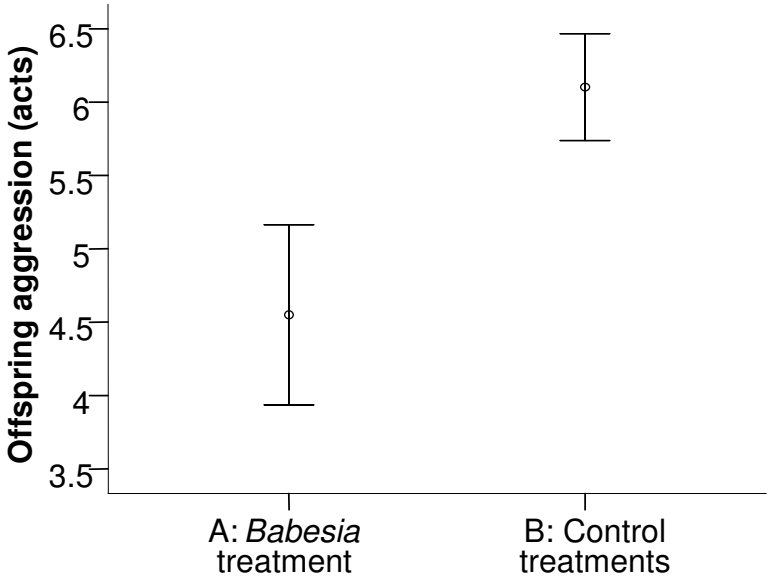


Figure 4.4: Treatment effects on adult offspring aggression. Measured as the total observed acts of aggression in novel social groups. Error bars represent ± 1 standard error (SE).

4.3.3 Effects on adult offspring response to disease challenge

No effect of socialising treatment on infection profile was found (Repeated measures GLM: $F_{5,134} = 0.612$, $P = 0.691$; PC1 Score GLM: $F_{1,\ 143} = 0.364$, $P = 0.547$). Therefore data from socialised and non-socialised offspring were pooled. Offspring from *Babesia*-treatment dams showed a different response to disease across the period of infection (Figure 4.5A: Repeated measures GLM: $F_{5,30} = 2.612$, $P = 0.045$). Principal components analysis of key features of the infection profile showed that this difference reflected an accelerated time course of infection, with offspring from *Babesia*-treatment dams showing earlier onset, peak and clearance of infection than

offspring from control dams (Table 4.3; Figure 4.5B: GLM: $F_{1,34}$ = 4.314, P = 0.045).

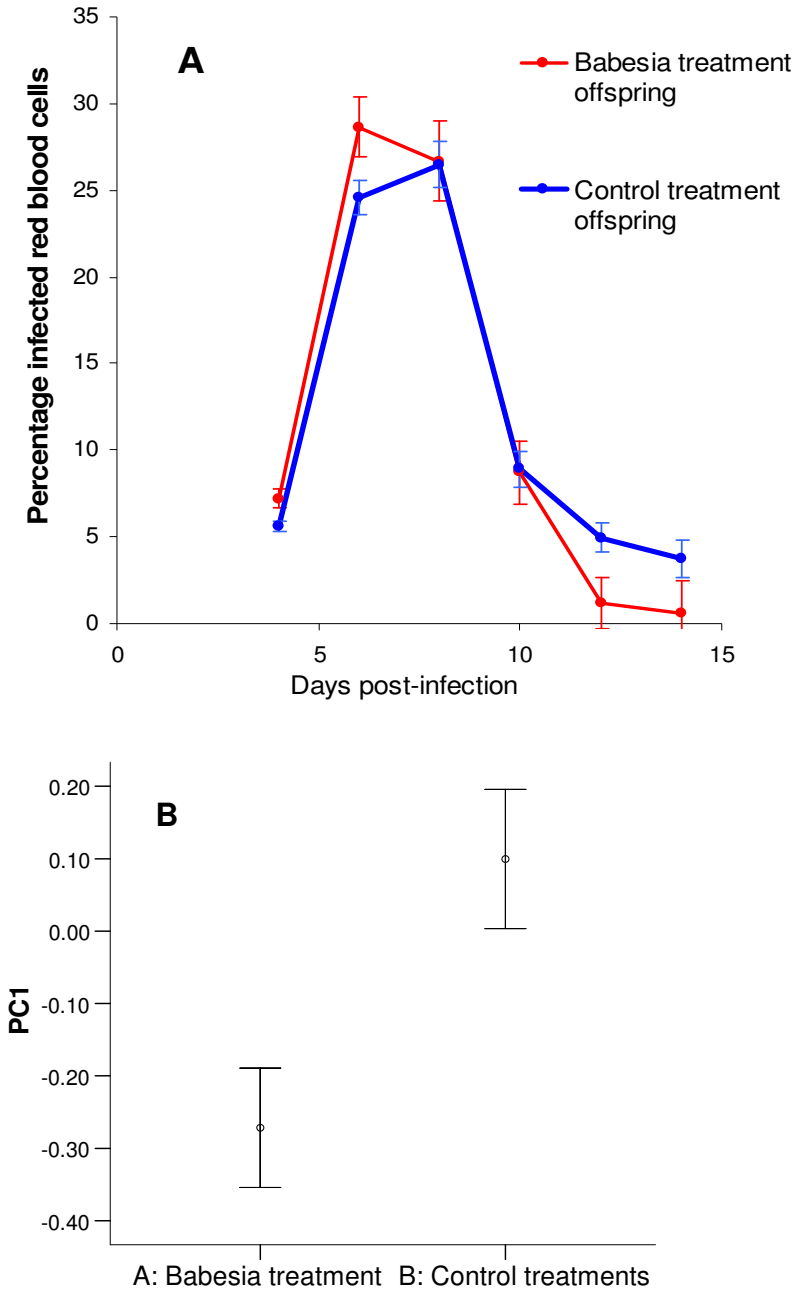


Figure 4.5: Treatment effects on adult offspring infection profiles. A, The time course of infection with *B. microti* in adult offspring of dams exposed during pregnancy to infected males (red line) or control males (blue line). **B,** Scores for the first component (PC1)

extracted by principal components analysis of five characteristics of the infection profile for offspring from (A) *Babesia*-treatment and (B) Control-treatment dams (see Table 4.3: high positive scores reflect later onset, peak and clearance of infection). Error bars represent ± 1 (SE).

Table 4.3: Principal component analysis of infection profile, showing loadings derived from analysis of the time course of the infection. Scores for different treatments are shown in Figure 4.5B.

Component 1	
Eigenvalue	2.652
% Variance explained	53.032
Loadings	
Day of onset of infection[a]	0.943
Day of peak of infection	0.921
Duration of infection (onset to clearance)[b]	0.724
Time to clearance (from inoculation)	-0.454
Infection level at the first clearance in the population	0.428

[a]Onset measured as the day on which infected red blood cells were first seen in blood smears.
[b]Clearance measured as the first day on which no red blood cells were infected following an infection peak.

4.4 Discussion

I found that pregnant dams housed in partitioned cages opposite infected conspecifics were able to detect information about the infection status of those individuals. This information caused physiological changes (elevated levels of serum corticosterone and increased kidney size) in these indirectly-challenged dams. Treatment also led to changes in the adult behaviour of offspring, with those that had developed in mothers exposed to ambient cues indicating threat of disease showing lower levels of aggression as adults. Most importantly, these same offspring then showed a different response to infection, peaking and clearing infection earlier than individuals that had developed in mothers which did not have diseased neighbours.

Corticosterone is the primary glucocorticoid found in rats and mice, and is homologous to cortisol in humans and many other mammals (Edwards & Burnham, 2001). Elevated levels of glucocorticoid reflect physiological changes associated with stressful stimuli (De Kloet *et al.*, 2005) including social information about distress (Boissy *et al.*, 1998) and immune responses (Fernandes, 2000) in conspecifics. Therefore the patterns seen here are consistent with the conclusion that the females had detected disease in neighbours. This detection did not appear to illicit an immune response in the *Babesia* treatment dams (there was no effect on IgG production or spleen growth). The unusual kidney growth seen is difficult to explain, but may be the result of the kidney's proximity to the adrenal gland, which was overacting to produce high levels of corticosterone in these females.

As the females in this study were exposed to auditory, behavioural, visual and olfactory information about their neighbours, a number of cues may have been used to detect disease in the stimulus males. There is no known effect of infectious disease on mouse vocalisations, but with increasing understanding of the complexity of mouse "song" (Holy & Guo, 2005) such a relationship may emerge, and may have provided an indicator for dams in this experiment. Behavioural cues may also have played a role, but *B. microti* at the inoculation levels I used has few recorded effects on host behaviour (Barnard *et al.*, 1996a), and I found no differences in behaviour between infected and uninfected stimulus males (see Table 4.2). The eyes and ears of laboratory mice become marginally paler at peak infection due to mild anaemia (Homer *et al.*, 2000), but it is unlikely that this would have a been an important cue given the poor eyesight of albino mice.

Disease status is perhaps most likely to have been assessed via olfactory cues. In experiments in which only conspecific urine is offered to subjects as a sensory cue, mice are able to detect infection (Kavaliers *et al.*, 2005a; Kavaliers *et al.*, 2003; Penn, 2006; Penn & Potts, 1998a; Penn *et al.*, 1998; Zala *et al.*, 2004). The underlying mechanism is currently unknown, but there are various hypotheses. Infections can change the composition of the commensal microbial community that plays an important role in individual odour (Hurst *et al.*, 2001; Lanyon *et al.*, 2007; Penn & Potts, 1998a). Infection also leads to increased expression of MHC molecules, and decreased MUP production, influencing the concentrations of volatile acids and other excreted endocrine by-products in the urine (Isserhoff *et al.*, 1986; Penn & Potts, 1998a). Antigens, antibodies and elevated levels of NO have been

found in the urine of human malaria patients (the malaria parasites *Plasmodium falciparum* and *P. vivax* are a similar intraerythrocytic parasites to *B. microti*) (Anstey *et al.*, 1996; Rodriguez-del Valle *et al.*, 1991). In my study, females may have also detected components of the parasites shed in the faeces, or subtle changes in the stimulus males' behaviour that I was not able to detect. Thus the females in this study had access to a broad range of social information with which they could potentially determine the disease status of their neighbours, with important consequences for their own physiology and ultimately the development of their offspring.

Offspring that developed in the *Babesia* treatment environment were less aggressive as adults. Pre- and post-natal maternal corticosterone levels have been shown to affect the behaviour of offspring as adults via *in utero* exposure through the blood or post-partum through milk (Breuner, 2008; Edwards & Burnham, 2001; Takahashi *et al.*, 1992). Improved learning, reduced anxiety, sleep disturbances, enhanced fear, reduced social interactions, and decreased exploratory behaviour have all resulted from endogenously or exogenously elevated corticosterone or cortisol during development (Catalani *et al.*, 2002; Catalani *et al.*, 2000; Catalani *et al.*, 1993; Edwards & Burnham, 2001; Glynn *et al.*, 2007; Mateo, 2008). In humans, maternal stress during pregnancy is associated with a range of physical and behavioural effects in children and adult offspring (Breuner, 2008; Edwards & Burnham, 2001; Gluckman *et al.*, 2005b; Rickard & Lummaa, 2007). It is therefore possible that the behavioural changes found among the adult offspring of *Babesia* treatment mothers were mediated by maternal corticosterone. Interestingly, other studies of the effects

of maternal stress on offspring have also found that behavioural changes are not evident before weaning in either the mother or offspring, but emerge later when the offspring reach adulthood (Casolini *et al.*, 1997), as was the case in this study (see Table 4.2).

Aggression in adult mice is associated with social dominance, territory acquisition and maintenance, and consequently increased access to mating opportunities (Barnard *et al.*, 1994; Meagher *et al.*, 2000; Waterman, 2007). However, there is evidence that the benefits of aggressive behaviour are counter-balanced by costs associated with reduced resistance to disease (Barnard *et al.*, 1994; Whitacre, 2001). In this study, offspring of dams exposed to the *Babesia* treatment environment showed both accelerated response to infection (particularly an accelerated resolution phase, see Figure 4.4), and reduced aggression in novel social groups. These results thus support the existence of a trade-off between social dominance and disease resistance. Evidence from other studies strongly implicates testosterone in the mediation of dominance-resistance trade-offs (Alexander & Stimson, 1988; Barnard *et al.*, 1994; Decristophoris *et al.*, 2007; Whitacre, 2001; Zala *et al.*, 2008a). Of particular relevance in the context of my study, testosterone is known to interact with CD4+ T cells (Roberts *et al.*, 2001), which are important for the resolution phase of *B. microti* infection (Homer *et al.*, 2000). However, as I was not able to detect a significant effect of treatment on testosterone levels (Table 4.2), I cannot confirm a direct role for this hormone in the trade-off that I appear to have observed. Nevertheless, my results do point to a role for maternal corticosterone in the response to exposure to diseased conspecifics. Elevated levels of corticosterone may have led

to the observed decrease in costly aggressive behavioural and the increased immunocompetence seen in the *Babesia* treatment offspring. These results are consistent with evidence from the literature on birds, where maternal hormones are increasingly recognised to be one of the most important factors mediating transgenerational immune priming (Groothuis *et al.*, 2005b; Müller *et al.*, 2005; Tschirren *et al.*, 2004).

I suggest that this study demonstrates adaptive investment in immunocompetence (Manz *et al.*, 2005) (clearing infection sooner), in a situation in which the imminent threat of infection has been perceived, possibly at the expense of investment in the acquisition of social dominance. Accelerated clearance of infection would return individuals to a competitive (Kilpimaa *et al.*, 2004) and attractive (Ehman & Scott, 2002; Feore *et al.*, 1997; Hamilton & Zuk, 1982) state more quickly, and thus enable them to secure future mating opportunities. Such benefits could outweigh any costs associated with reduced social dominance in an environment where the risk of disease is high. Thus, I conclude that either dams (through strategic maternal investment (Marshall & Uller, 2007)) or offspring (through individual life-history "decisions") responded adaptively to an ambient threat of infection in order to maximise the chances of offspring survival (Hazel *et al.*, 2000), and ultimately reproductive success.

To my knowledge, this study provides the first evidence for transgenerational regulation of immunocompetence based on social information, and the implications of the findings are wide-reaching. These results are of great significance for our

understanding of the role of parasites in the evolution of life histories (Virgin, 2007), adding maternal perception of disease risk in the immediate environment to the factors potentially determining future social dominance, and related aspects of fitness, in offspring. Furthermore, the individual differences in disease susceptibility found across species, including humans (Bateson *et al.*, 2004; Rickard & Lummaa, 2007), might be explained in part by similar maternal effects (Zinkernagel, 2000). Through immunological maternal effects, individual decisions may have population level consequences in following generations (Mitchell & Read, 2005), adding a new complexity to our understanding of epidemiological processes.

These findings have implications for both animal welfare and the validity of scientific procedures. Most animal housing units enable some level of auditory and olfactory interaction between individuals (ASPA, 1989). Here, I have shown that a measurable perception of disease in neighbouring conspecifics occurs, and elicits a stress response in the perceiver. Therefore the results highlight the potential for substantial and unexpected effects of experimental design on animal welfare (Barnard & Hurst, 1996; Leach *et al.*, 2008). Given this, I support the call for animal welfare sections in publications (Würbel, 2007) with the added suggestion that the welfare of bystanders, as well as experimental subjects, should be considered when planning experimental work. Finally, I question the accuracy of considering co-housed "control" animals as "untreated" in scientific procedures, when this experiment has shown that they can respond in complex physiological and behavioural ways to ambient information from their treated neighbours.

Chapter 5: Direct immune challenge experiment

5.1 Introduction

Chapter 4 showed that female mice exposed to ambient immunological information produced offspring that were more resistant to disease and less aggressive as adults. Here, I investigated transgenerational transmission of immunological information by directly infecting dams prior to pregnancy (rather than co-housing them with infected individuals). This meant that dams had direct information about infectious agents in the environment, and it added the complexity of dams having potentially reduced resources due to recent infection. I examined how recent disease affected reproductive output and maternal behaviour in dams, and how it affected the social behaviour and disease resistance of their offspring as adults.

5.1.1 The long reach of the parasite phenotype

Direct infection of dams (as opposed to infection of neighbouring conspecifics) complicates the potentially adaptive pathways involved in transgenerational effects of infection, because the parasite itself becomes a major player in the evolutionary struggle. Parasite-induced changes in host phenotype are well documented (Poulin & Thomas, 2008; Thomas *et al.*, 2005). Parasites often change the phenotype (e.g. the morphology, physiology or behaviour) of their hosts in order to maximise the likelihood of transmission to new hosts (Thomas *et al.*, 2005). In particular, trophically transmitted parasites are known to alter the behaviour of their intermediate hosts so as to make them more vulnerable to predation by the parasites' ultimate hosts (Lafferty, 1999; Moore & Gotelli, 1990; Seppälä & Jokela, 2008; Thomas *et al.*, 2005). For example, *Toxoplasma gondii* infected rats visit areas with high cat predation risk more often, increasing the likelihood that the protozoan will reach its definitive host (Berdoy *et al.*, 2000). Killifish (*Fundulus pinniparvus*) display increased levels of conspicuous behaviour when infected with larval trematodes, making them significantly more susceptible to predation by birds (Lafferty & Morris, 1996). Non-trophically transmitted parasites also manipulate host behaviour to aid transmission. For example, the parasitic fungus *Entomophthora muscae* causes its insect hosts to die perched in a position favourable to wind dispersal of spores (Maitland, 1994).

Parasitic manipulation of host phenotype includes manipulation of reproductive behaviour and output (Haine *et al.*, 2007; Kristan, 2002a; Poulin & Thomas, 2008;

Willis & Poulin, 1999). Microsporidians pass vertically from mother to offspring through the cytoplasm of the ova, but cannot pass into sperm (Kelly *et al.*, 2003). Therefore, male hosts represent a dead end for such parasites. Given this, microsporidians manipulate the sex ratio of host offspring towards more daughters to maximise their own transmission (Dunn *et al.*, 1996; Kelly *et al.*, 2003; Mautner *et al.*, 2007). *Wolbachia* bacteria similarly increase cytoplasmic vertical transmission by causing feminisation or parthogenesis in their various arthropod hosts (Bouchon *et al.*, 1998; Jiggins *et al.*, 2000). In these cases of vertical transmission, it is easy to see how the strong selective pressure for optimising host reproductive outputs would arise, as the parasite is entirely dependent on this mode of transmission. It is, of course, possible that parasites in the population would benefit from manipulating nearby currently uninfected potential hosts. Such manipulation has never been reported, but would add yet more complexity to the interactions seen in Chapter 4.

Parasites have been shown to affect reproductive investment and the consequent behaviour and life history of offspring even when vertical transmission does not exist (Cezilly & Perrot-Minnot, 2005). However, it is often difficult to disentangle whether these effects reflect: a) adaptive manipulation beneficial to the parasite (Chadwick & Little, 2005; Kelly *et al.*, 2003; Mautner *et al.*, 2007), b) inevitable side-effects of the cost of combating disease (Hakkarainen *et al.*, 2006; Whitaker & Fair, 2002), or c) adaptive responses to infection by the host ensuring their own reproductive success and that of their offspring (i.e. parasite-mediated maternal effects) (Cezilly & Perrot-Minnot, 2005; Poulin, 1995; Poulin & Thomas, 2008; Rolff, 1999). Flea-infested great-tits produce fledglings which disperse shorter

distances and are likely to be recruited to the local population, which would potentially be to the benefit of the parasite by providing nearby future hosts (Heeb *et al.*, 1999). Poulin and Thomas (2008) discuss epigenetic mechanisms by which this transgenerational manipulation might be achieved. In such a situation, the extended phenotype (Dawkins, 1982) of the parasite can be considered to extend across two host generations. On the other hand, mite-infected female lizards (*Lacerta vivipara*) display what appears to be an adaptive parasite-mediated maternal effect - a response that is beneficial to the host. They produce offspring which develop faster, invest more in early reproduction, and run faster: literally and figuratively living faster and dying younger in an environment when life-expectancy is reduced (Sorci & Clobert, 1995; Sorci *et al.*, 1994).

5.1.2 Trans-generational immune priming

One particular kind of parasite-mediated maternal effect, trans-generational immune priming (henceforth TGIP), occurs in a wide range of taxa (Agrawal *et al.*, 1999; Gallizzi *et al.*, 2008). Wild radish plants (*Raphanus raphanistrum*) display a form of TGIP; mature individuals which experience high levels of insect herbivory (which is in many ways similar to animal parasitism) producing seedlings with increased pest resistance (Agrawal, 2002). In animals, maternal exposure to pathogens or antigens (e.g. bacterial polysaccharides) is shown to promote offspring immunocompetence in species ranging from insects to birds to mammals (Grindstaff *et al.*, 2006; Kristan, 2004; Moret, 2006). Queen bumblebees (*Bombus terrestris*) injected with bacteria produce workers which show higher levels of antibacterial activity (Sadd *et al.*,

2005). Immunised pied flycatchers (*Ficedula hypoleuca*) produce offspring with elevated endogenous antibody production (Grindstaff *et al.*, 2006). Offspring from house mouse dams infected with an intestinal nematode when pregnant are more likely to clear infection as adults (Kristan, 2002b; 2004).

The mechanisms involved in TGIP are still unclear. Some instances appear to involve optimising normal reproductive behaviour to benefit offspring (e.g. skewing laying order relative to offspring gender if one sex is more vulnerable to disease (Badyaev *et al.*, 2006)). Others involve direct transfer of antibodies or immunoglobulins through the milk or yolk (Hanson *et al.*, 2003). A third group can be considered epigenetic effects, whereby maternal experience affects DNA expression, but not sequence, in offspring (Poulin & Thomas, 2008). This may be achieved by differential exposure of offspring to androgens and other hormones during development (Grindstaff *et al.*, 2006; Groothuis *et al.*, 2005b; Sobrian *et al.*, 1992; Tschirren *et al.*, 2004), or by differential methylation of DNA (either somatic or gametic) through other biochemical pathways (Jones & Takai, 2001; Poulin & Thomas, 2008).

While evidence for TGIP is growing, it remains difficult to determine the causal factors – many previous studies involve females that are infected at the time of pregnancy/egg laying. Often the responses found are attributed to whichever causal agent (host or parasite) would intuitively benefit from the responses found. In addition, the downstream effects and potential costs of phenotypically flexible pre-emptive investment in immunocompetence have not yet been explored (Hamilton &

Zuk, 1982; Lochmiller & Deerenberg, 2000; Marshall & Uller, 2007; Roberts *et al.*, 2001).

5.1.3 A direct immunological challenge experiment

In this experiment I aimed to explore transgenerational effects of a parasitic infection in the mother, rather than foetal immunological responses to direct challenge. Therefore it was important that offspring were not directly infected by vertical transmission (New *et al.*, 1997). In addition, I was interested in adaptive maternal responses, rather than the long reach of the parasitic phenotype. As in Chapter 4, BKW mice and *B. microti* were used. In mice, *B. microti* induces high but transient parasitemias, which are quickly cleared (Homer *et al.*, 2000), and so it provides an excellent parasitic tool for investigating the transgenerational effect of mild disease. Babesial agents have been shown on occasion to pass transplacentally from mother to offspring *in utero* (Kjemtrup & Conrad, 2000; New *et al.*, 1997). For example, *B. bovis* in cattle, *B. canis* in dogs, *Theileria equi* (formerly *Babesia equi*) in horses and *B. microti* in humans have all been shown to pass through the placenta (Feder *et al.*, 2003; Kjemtrup & Conrad, 2000; New *et al.*, 1997). Therefore, unlike the experiment described in Chapter 4, when the immunological stimulus was given to dams during pregnancy, here I chose to treat dams prior to mating. I also ensured that all infected dams had cleared infection prior to mating and hence before pregnancy. I further validated that offspring were not infected by taking smears at weaning. Serum immunological constituents and response to disease later in life confirmed the absence of infection in new born offspring (see Results). By eliminating the risk of

vertical transmission, I limited the extent to which responses which were seen in offspring might be the product of parasite-beneficial manipulation, although this possibility still cannot be excluded.

Infecting dams prior to, rather than during, pregnancy had additional welfare-related benefits. *B. microti* can cause mild anaemia and associated lethargy and weight loss in mice (Homer *et al.*, 2000), and I wished to avoid compounding any adverse effects of the disease by separating the stress of peak infection from the metabolically demanding process of gestation (Feder *et al.*, 2003).

5.1.4 Babesia *and pregnancy*

Other than documented cases of vertical transmission, and foetal abortion due to babesiosis during pregnancy (Kjemtrup & Conrad, 2000), there is very little information in the literature regarding the impact of peri-natal *Babesia* infection on offspring. More research has been done on the consequences of the similar malarial parasites, *Plasmodium falciparum* and *P. vivax*, which are very similar to *Babesia spp.* but a much more significant cause of serious disease and high mortality in humans (Krause *et al.*, 2007; Marzal *et al.*, 2005). Research has shown that humans born to malarial mothers have lower birth weights due to inter-uterine growth retardation. They also tend to be premature, and there is an associated increased risk of stillbirth (Menendez *et al.*, 2000; Nosten *et al.*, 1999; Steketee *et al.*, 1996). However, most of these studies of malaria investigate the implications of peri-natal parasitaemia from a human health perspective. Very few offer insight into offspring

life-history consequences of maternal infection (but see Marzal *et al.*, 2005), or into the potentially adaptive offspring disease resistance due to maternal exposure prior to pregnancy.

Here, I will use a system where the risk of parasitic manipulation or vertical transmission is largely removed by ensuring that no pathogen is present at the time of conception. I will also examine the downstream life-history consequences of any TGIP by monitoring the physiology and social behaviour of offspring as adults, as well as their response to disease.

5.2 Methods

Methods overview

Figure 5.1 details the plan of experimental work. BKW mouse dams were either infected with the blood protozoan *B. microti*, or subject to one of four control treatments. Following clearance of infection, dams were co-housed with sire males for six days. Mated dams were then removed to single housing cages until parturition. At 11 days of age each litter was reduced to four males. Dams were removed from offspring at 24 days of age. At 50 days old approximately half of the offspring were re-housed with three novel males from the same treatment group. Continuous behavioural observations totalling 200 minutes per group were carried out, recording aggressive behaviour in the adult offspring. At 70 days of age the adult offspring were singly housed and infected with *B. microti*. The time course of the infection was monitored by blood smears until clearance. Blood was taken from dams and offspring at various time points during the experiment (Figure 5.1) and assayed for testosterone, corticosterone and total IgG.

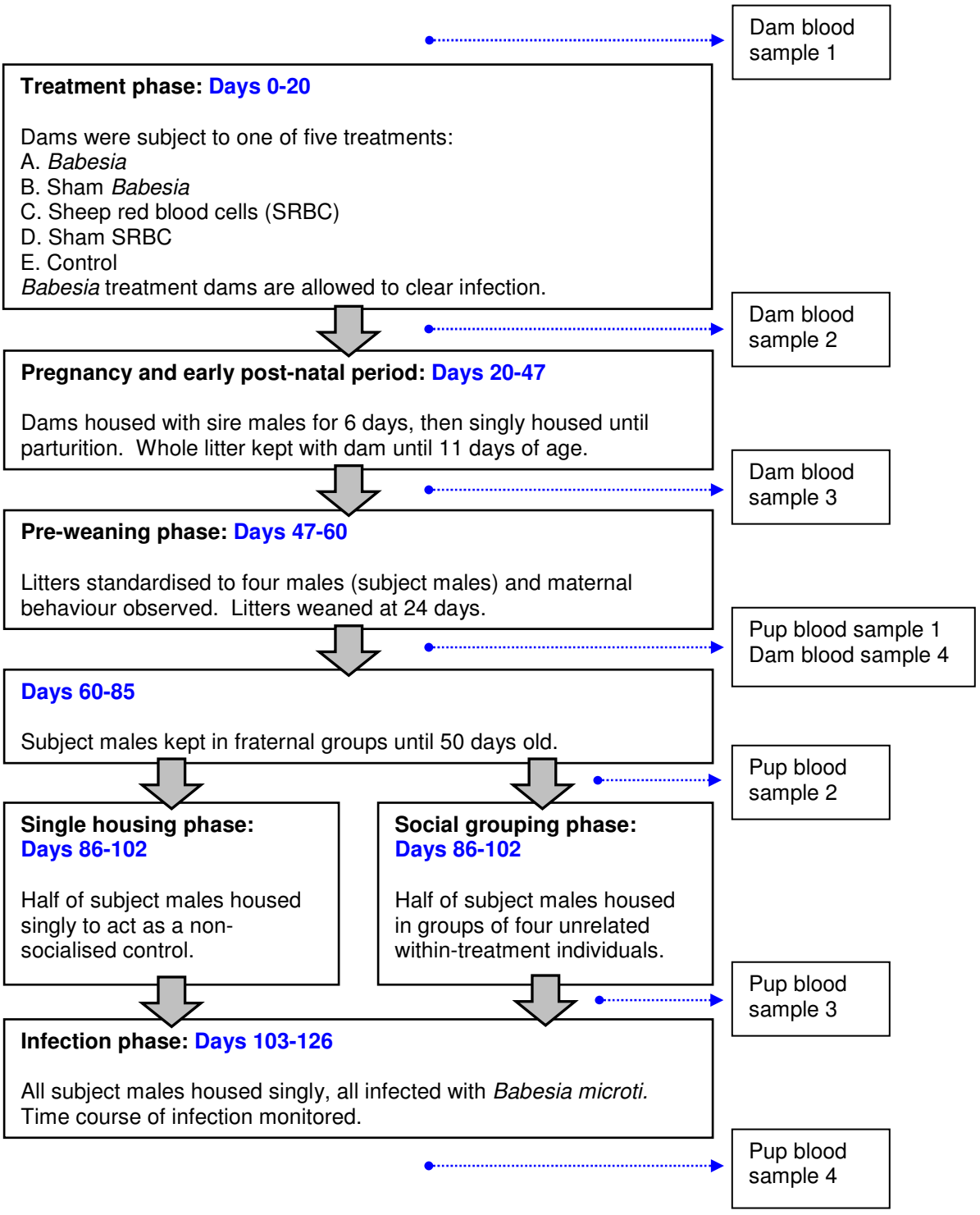


Figure 5.1: Plan of experimental work including timings of blood samples.

Detailed Methods

5.2.1 Mice and housing

A priori power tests were conducted to determine minimum sample sizes necessary to detect treatment effects of a magnitude that would be considered significant in order to minimise animal use. The subjects were 358 mice of the BKW strain. This number comprised 75 subject dams (seven weeks old), 75 sire males (nine weeks old) and 208 male offspring from the subject dams (from birth to 90 days of age). Subjects were kept in standard cages throughout (see General Methods).

5.2.2 Treatment phase

Seven week old dams were randomly divided into five treatment groups of 15 individuals each. These were:

A. ***Babesia* treatment:** Infection with 1 x 10^7 red blood cells harbouring *B. microti.*

B. **Sham *Babesia*:** Sham infection of *B. microti,* comprising only the Hank's solution used to suspend erythrocytes in Treatment A.

C. **SRBC treatment:** Inoculation with 5 x 10^7 SRBCs, which would elicit an immune response, but no pathology.

D. **Sham SRBC:** Sham inoculation of SRBCs, comprising only the Hank's solution used to suspend the SRBCs.

E. **Complete control:** No inoculation, but Treatment E dams were handled, monitored and sampled in the same way as all other dams.

Inoculations and infections followed the protocols detailed in the General Methods. This phase lasted 20 days, by which time all of the infected dams (*Babesia* treatment) had peaked and cleared infection.

5.2.3 Mating phase

The dams were then introduced to the home cages of randomly allocated single-housed sire males, and kept in these pairs for six days. This method was used to minimise sire-dam aggression, and maximise likelihood of impregnation (Koyama, 2004). After six days, dams were removed from sire cages and housed singly to continue pregnancy and then give birth. They were then kept with full litters for 11 days.

5.2.4 Pre-weaning phase

At 11 days of age, all pups were sexed and each litter reduced to four males (three males in the case of three litters that had only three males each) for reasons explained in the Chapter 4 Methods (Section 4.2.4). A total of 208 male pups were included in the following stages of the experiment, from 52 females. Thirteen dams did not become pregnant in the mating phase, and a further ten dams did not have at least three sons at eleven days post-partum, but neither the likelihood of becoming pregnant nor litter sex ratio were affected by treatment (see Results). At 24 days of age all mothers were removed from litters, and pups (subject males) were left in their fraternal groups until 50 days old.

5.2.5 Social grouping and single housing phases

At 50 days of age all subject males were separated from their siblings. Approximately half of the subject males (100 mice) were randomly chosen and re-housed with three novel males from the same treatment group, with whom they were allowed to establish dominance hierarchies. Continuous behavioural observations totalling 200 minutes (10 minutes per day) per group were carried out. Various social interactions were recorded (detailed in General Methods Table 2.2), in order to determine social rank within these groups. The remaining half (108 mice) was housed singly to act as a non-socialised treatment. At the end of this phase all males were removed and housed singly in new clean cages.

5.2.6 Infection phase

At 70 days of age the adult offspring, all now housed singly, were injected with 1 x 10^7 red blood cells infected with *B. microti*. The time course of the infection was closely monitored until clearance (for details of monitoring procedures, see General Methods).

5.2.7 Technical procedures

All monitoring, sampling and handling of dams in Treatments A and C were repeated accordingly on dams in Sham Treatments B, D and E. Sham infection and inoculation involved the introduction of Hanks' solution only. All subject offspring were infected in a random order with 1 x 10^7 infected red blood cells of *Babesia.* *Babesia* infection was monitored by blood smears, and hormonal and IgG levels

were monitored by 50µl blood samples at various time points (see Figure 5.1). In a number of cases, limited serum volumes precluded reliable estimates for all three serum factors at all time points from certain individuals. As a consequence, sample sizes of some analyses vary. For details of blood sampling and assay procedures see General Methods.

5.2.8 Statistical analysis

All analyses were based on pooled control treatment groups B-E as no significant differences were found between these groups (see Results section, Table 5.1). Where appropriate, values were nested within dam (which was included as a random factor), or averaged per dam to avoid within-litter pseudoreplication. Principal components analysis conducted on five correlated variables describing individual infection profiles generated a single principal component which captured the majority of the variation in the characteristics of the profiles (PC1). Individual scores for this component were then used in further analysis. In addition to sample size variation due to blood sampling limitations, sample sizes may vary among early phase analyses (for example, changes in dam physiology over infection phase, compared to offspring measures) because not all females became pregnant or produced four sons.

5.3 Results

5.3.1 Physiological responses to treatment in the dam

As there were no significant differences found among the four control treatments for the response variables analysed, (Table 5.1), these were grouped as one treatment labelled "control treatments" and compared against the *Babesia* treatment dams.

Table 5.1: No differences were found among the four control treatments (see Methods) in the following physiological and behavioural measures.

	F	*Degrees of Freedom*	*P - value*
Weight changes over pregnancy [b]	0.280	6, 74	0.944
Dam suckling behaviour [a]	1.485	3, 39	0.234
Dam $\log_{10}$(IgG) levels over experiment [b]	0.591	9, 81	0.816
Offspring weight in early adulthood [b]	1.305	9, 492	0.231
Offspring suckling behaviour [a]	1.756	3, 39	0.172
Offspring $\log_{10}$(IgG) at weaning [a]	1.547	3, 39	0.218
Offspring $\log_{10}$(IgG) at 49 days of age [a]	1.886	3, 40	0.147
Offspring testosterone change over grouping [a]	1.294	3, 47	0.287
Offspring infection profile [b]	1.450	15, 195	0.128
Weight loss over infection [a]	0.919	3, 40	0.440
Change in IgG over infection [b]	0.282	3, 112	0.839

[a] GLM
[b] Repeated measures GLM of measurements taken across experiment

Babesia treatment dams showed significantly higher levels of serum IgG throughout the experiment following infection (Figure 2, Repeated measures GLM on $\log_{10}$ (IgG): $F_{3,105} = 39.983$, $P < 0.0001$). There was no treatment effect on serum corticosterone (Repeated measures GLM: $F_{3,147} = 0.283$, $P = 0.838$). *Babesia* treatment dams lost significantly more weight over the period of infection than dams in all other control groups (GLM: $F_{1,72} = 7.517$, $P = 0.008$), but differences at the time of mating were of marginal significance (GLM: $F_{1,72} = 3.884$, $P = 0.053$). Body weight at mating did not appear to have significant downstream effects (effect on

likelihood of becoming pregnant: GLM: $F_{1,72} = 1.272$, $P = 0.263$, relationship with litter size: Pearson's correlation: $n = 60$, $r = -0.203$, $P = 0.119$).

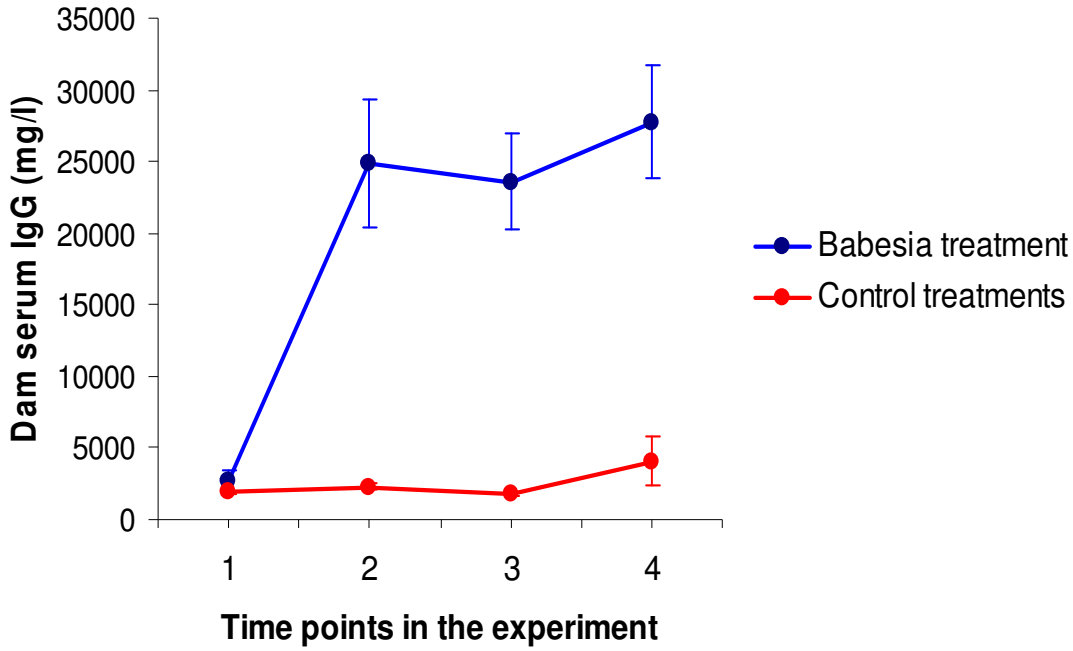


Figure 5.2: Dam serum immunoglobulin. Measured at the start of the experiment (1), following clearance of infection and prior to mating (2), at the end of pregnancy (3) and upon weaning (4). Error bars represent ± 1 standard error.

5.3.2 Reproductive responses to treatment in the dams

Treatment did not affect the likelihood of becoming pregnant (Chi-Squared test: $X^2 = 0.538$, $df = 1$, $P = 0.970$) or producing a live litter (Chi-Squared test: $X^2 = 0.397$, $df = 1$, $P = 0.983$). Nor did it affect the litter size (GLM: $F_{1,60} = 0.031$, $P = 0.831$) or the sex ratio of litters (GLM: $F_{1,57} = 0.056$, $P = 0.813$). However, the weight-gain over pregnancy, and weight-loss at parturition, was significantly affected by treatment. *Babesia* treatment dams gained more weight during pregnancy and lost more at

parturition, even when weight loss during infection and litter size were controlled for (Figure 5.3, Repeated measures GLM: $F_{2,116} = 6.566$, $P = 0.002$). *Babesia*-treatment dams then spent significantly more time suckling offspring than control dams (Figure 5.4, GLM: $F_{1,51} = 5.940$, $P = 0.018$).

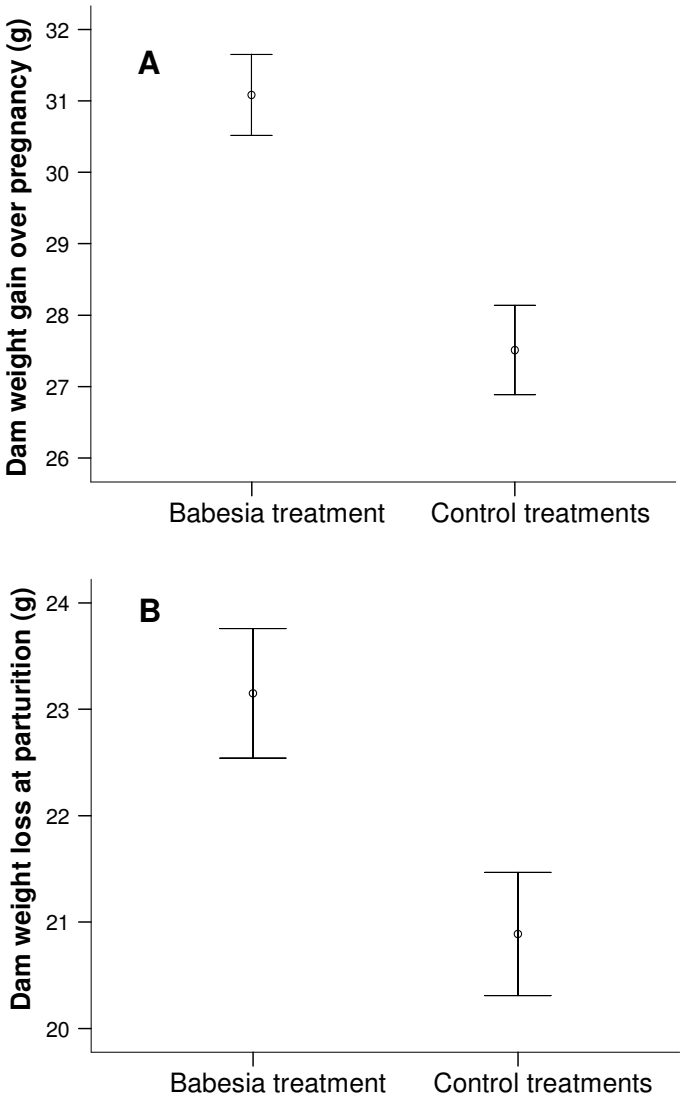


Figure 5.3: Dam weight changes over pregnancy and parturition. A. shows weight gain over pregnancy, B. shows weight loss at parturition. Error bars represent ± 1 standard error.

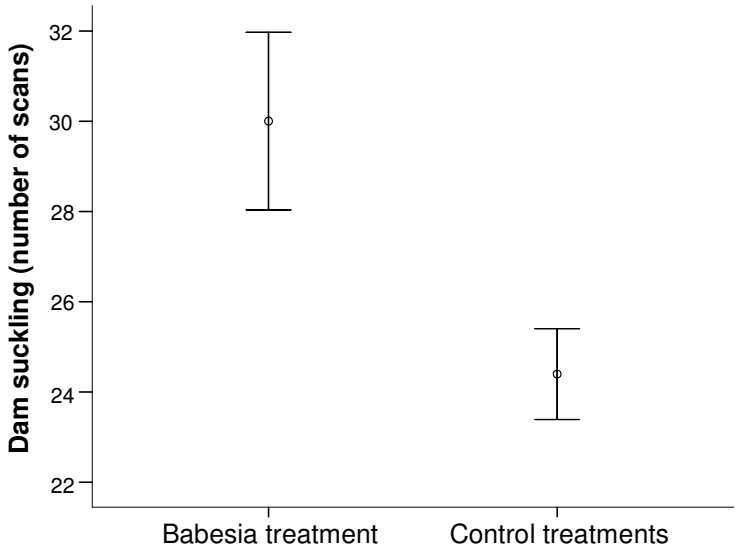


Figure 5.4: Suckling behaviour in dams. Total number of instantaneous scans in which dams were seen suckling one or more pup. Error bars represent ± 1 standard error.

5.3.3 Responses to treatment in the young offspring

There was no effect of treatment on early pup growth (Repeated measures GLM: $F_{2,114} = 0.818$, $P = 0.444$) but by weaning *Babesia* treatment pups were significantly lighter than control pups (Figure 5.5A: GLM: $F_{1,51} = 4.914$, $P = 0.031$). This was particularly surprising as *Babesia* treatment pups spent significantly more time suckling (Figure 5.5B: GLM: $F_{1,51} = 6.806$, $P = 0.012$). The effect of treatment on weight was no longer evident by five weeks of age, or thereafter (Repeated measures GLM: $F_{3,159} = 0.149$, $P = 0.930$).

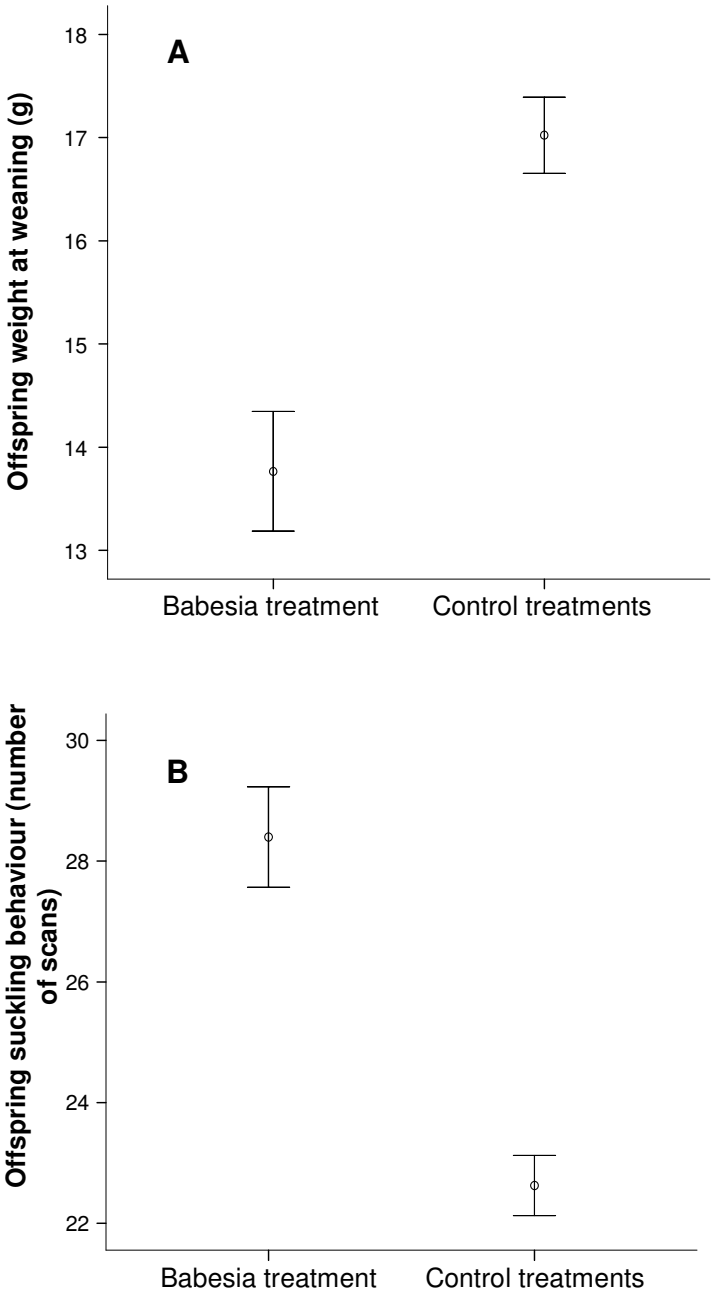


Figure 5.5: Growth and suckling behaviour of offspring. A: Offspring weight at weaning (24 days of age), B: Total number of instantaneous scans in which offspring seen suckling. Error bars represent ± 1 standard error.

Offspring from *Babesia* treatment dams showed significantly higher levels of IgG at weaning (Figure 5.6A: GLM on $\log_{10}$(IgG): $F_{1,53} = 17.529$, $P < 0.001$), a difference which persisted to seven weeks of age (Figure 5.6B: GLM on $\log_{10}$(IgG): $F_{1,53} = 9.612$, $P = 0.003$). There was no effect of treatment on early offspring corticosterone or testosterone (Repeated measures GLMs: corticosterone: $F_{3,153} = 0.423$, $P = 0.737$; testosterone: $F_{3,159} = 0.647$, $P = 0.586$).

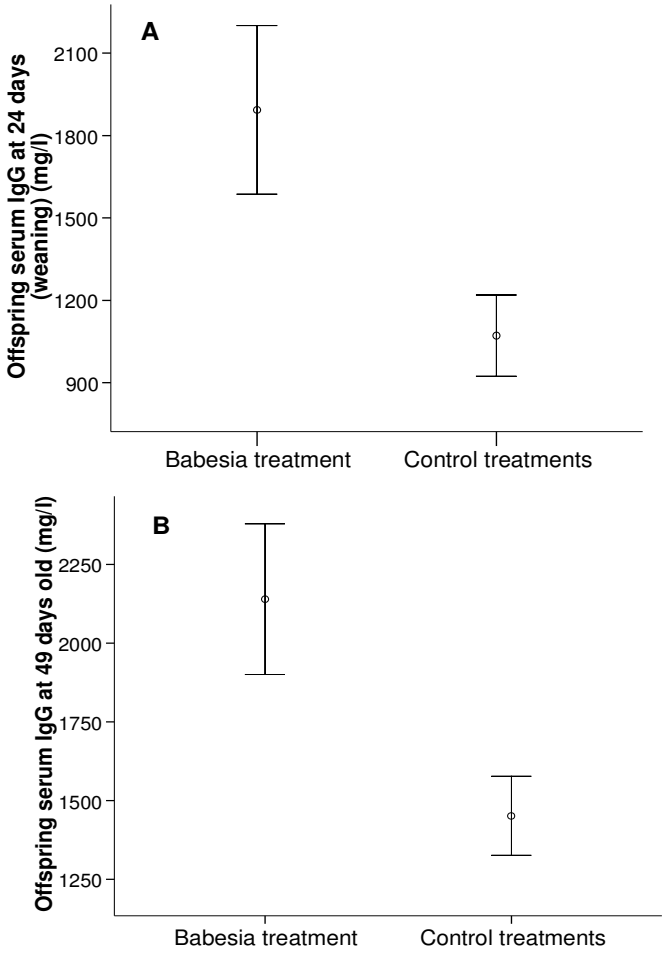


Figure 5.6: Offspring serum immunoglobulin. Measured in mg/l at A, 24 days (weaning) and B, 49 days of age (just prior to novel social grouping). Error bars represent ± 1 standard error.

5.3.4 Responses to treatment in the adult offspring

Over the social grouping phase, when the now adult males for the first time met non-sibling conspecifics, offspring from control treatments showed an increase in testosterone production, where *Babesia* treatment males showed a marked drop (Figure 7: GLM of change in serum testosterone: $F_{1,44} = 6.296$, $P = 0.016$). There was no such effect on corticosterone or immunoglobulin over the novel social group period (GLMs: change in corticosterone: $F_{1,49} < 0.001$, $P = 0.993$; change in IgG: $F_{1,48} = 1.206$, $P = 0.278$). Despite the effect on testosterone, there was no observable effect of treatment on social behaviour in adult offspring (√(Total aggression), GLM: $F_{1,56} = 0.247$, $P = 0.622$).

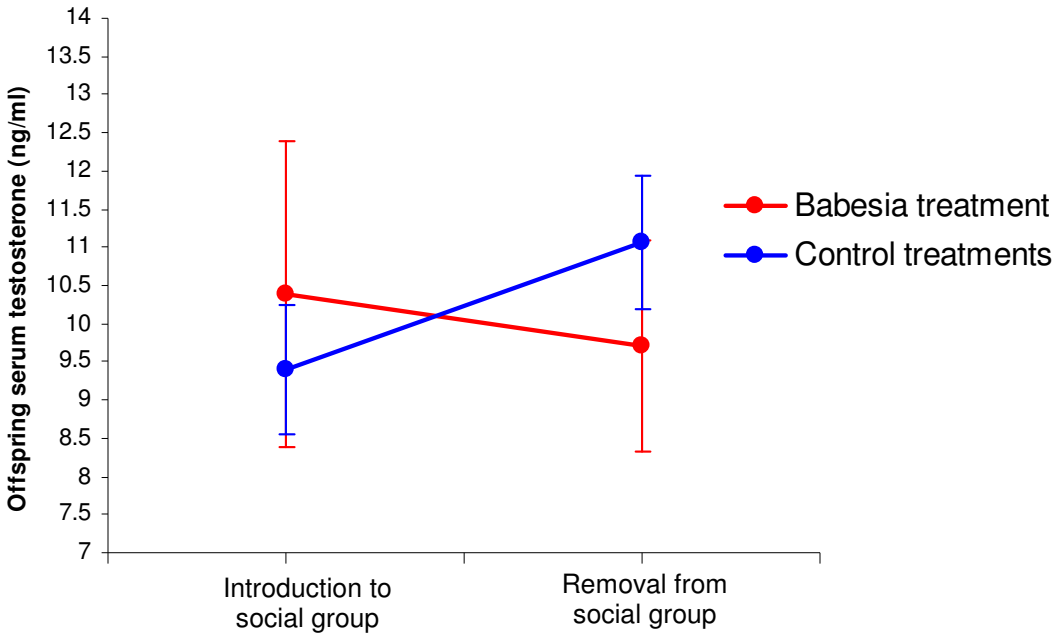


Figure 5.7: Changes in offspring testosterone. Serum testosterone measured on introduction to (49 days old) and removal from (65 days of old) novel social group caging as adults. Error bars represent ± 1 standard error.

5.3.5 Effect of treatment on the response of adult offspring to disease

Offspring from *Babesia* treatment dams had a significantly different response to *B. microti* infection as adults (Repeated measures GLM: $F_{5,255} = 3.314$, $P = 0.006$) with a faster resolution phase of the infection profile (Figure 5.8). This was not reflected in a PCA analysis of infection profile (Table 5.2, GLM of individual scores: $F_{1,48} = 2.002$, $P = 0.163$) (see previous chapter for further details of PCA). However, the accelerated response to disease was reflected in *Babesia* treatment males losing less weight over the infection phase (Figure 5.9, GLM: $F_{1,48} = 6.181$, $P = 0.016$). Interestingly, *Babesia* treatment males showed less of an increase in IgG over infection, even when early IgG levels were controlled for (Figure 5.10: Repeated measures GLM on $\log_{10}$ (IgG): $F_{1,51} = 6.197$, $P = 0.016$).

Table 5.2: Principal component analysis of infection profile, showing loadings derived from analysis of the time course of the infection.

Component 1	
Eigenvalue	2.435
% Variance explained	48.6
Loadings	
Day of onset of infection[a]	-0.095
Day of peak of infection	0.749
Duration of infection (onset to clearance)[b]	0.708
Time to clearance (from inoculation)	0.888
Infection level at the first clearance in the population	0.758

[a]Onset measured as the day on which infected red blood cells were first seen in blood smears. [b]Clearance measured as the first day on which no red blood cells were infected following an infection peak.

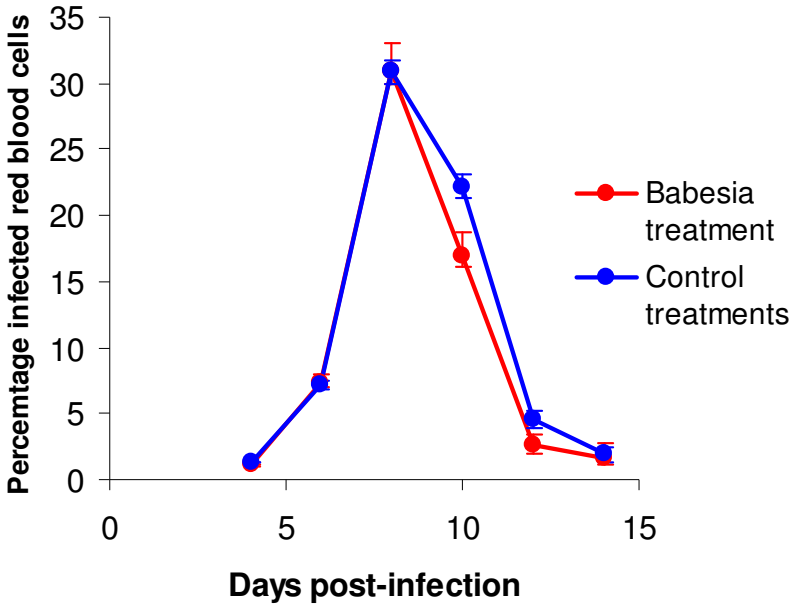


Figure 5.8: Offspring infection profiles. Percentage of red blood cells infected with *Babesia microti* at various time points following intraperitoneal injection with 1 x 10^7 infected red blood cells. Error bars represent ± 1 standard error.

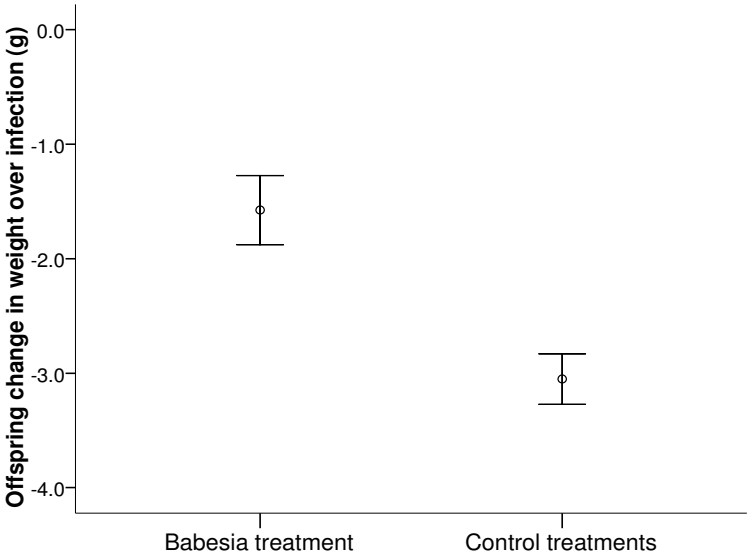


Figure 5.9: Offspring change in weight over infection. Difference between weight upon infection with *B. microti* by intraperitoneal injection, and the end of the experiment, (16 days later), following clearance of infection. Error bars represent ± 1 standard error.

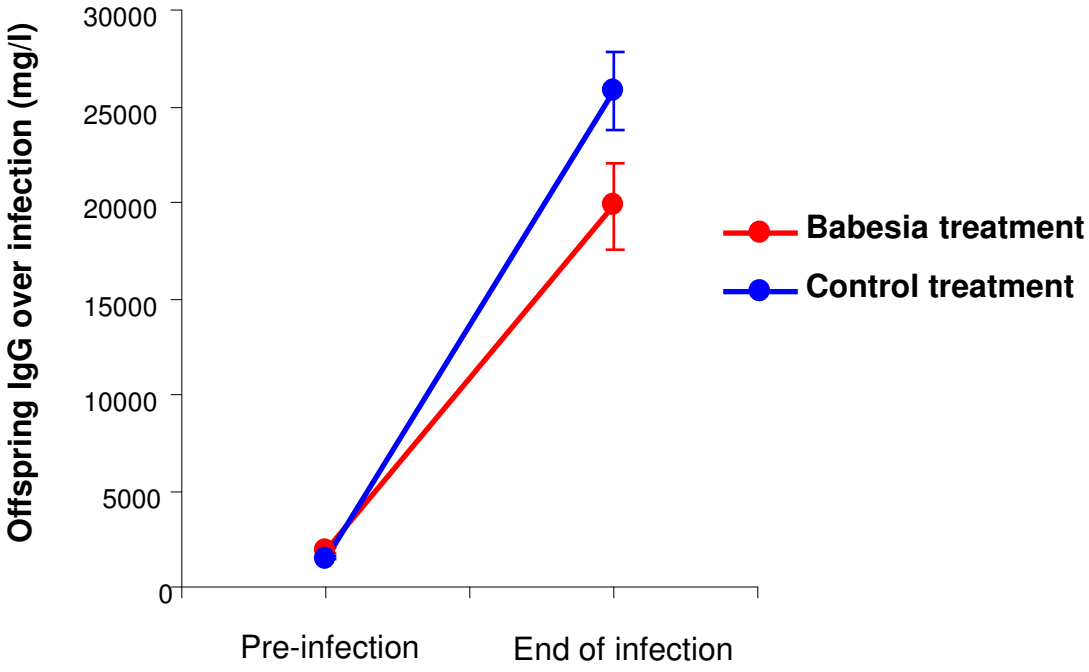


Figure 5.10: Change in offspring IgG over infection. Serum immunoglobulin measured prior to infection with $1x10^7$ infected red blood cells, and following clearance of the infection 16 days later. Error bars represent ± 1 standard error.

There was no overall effect of social grouping on infection profile (Repeated measures GLM: $F_{5,925} = 0.122$, $P = 0.988$), but males which were grouped and displayed the most aggression in their groupings (dominants) suffered higher infection peaks (Figure 11: GLM: $F_{1,98} = 4.172$, $P = 0.044$) than males which were less aggressive in groupings (subordinates). Treatment did not interact with the effect of rank on peak infection (GLM: $F_{1,96} = 0.004$, $P = 0.947$). Individuals of different rank did not show significantly different patterns of testosterone production over lifetime (Repeated measures GLM: $F_{3,276} = 0.518$, $P = 0.671$). No significant

interaction between rank and treatment on testosterone production over lifetime was found (Repeated measures GLM: $F_{3,270} = 1.883$, $P = 0.133$).

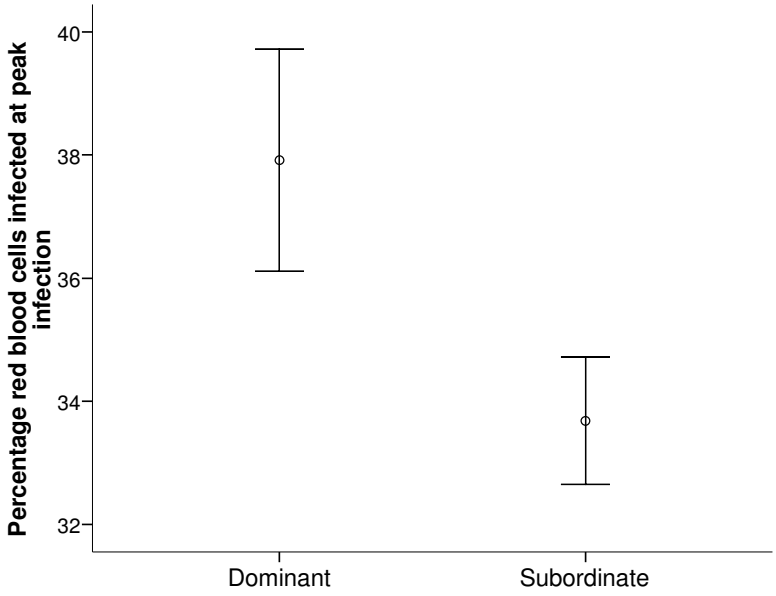


Figure 5.11: The effect of social rank on infection peaks. Offspring grouped in cages of four were ranked as dominant (one per cage) or subordinate (three per cage) based on their aggressive behaviour. Graph shows subsequent infection levels at the peak of infection. Error bars represent ± 1 standard error.

5.4 Discussion

This experiment aimed to examine how recent disease affects reproductive output and maternal behaviour in female mice, and offspring social behaviour and disease resistance as adults. The results support the findings of the previous chapter, where dams were indirectly exposed to disease by co-housing with non-contagious conspecifics. In both experiments, dams exposed to immunological cues had physiological responses to their exposure, and went on to produce offspring that showed an accelerated response to disease as adults, and differed in their responses (either behaviourally or hormonally) to novel social environments.

When working with live parasites (even if infection has been cleared) one is perhaps never able to remove the possibility that responses seen are driven by parasitic manipulation, as discussed in the introduction of this chapter. However, given the experimental schedule, and the direction of the responses seen in both dams and offspring here, it seems very unlikely that parasitic manipulation is responsible for these findings. Instead, the findings here support the conclusion that dams invested in reproduction in such a way as to maximise offspring resistance at a time when the risk of disease was high.

Following infection with *B. microti,* both dams and their offspring showed elevated levels of IgG. In natural systems, *Babesia* species are transmitted into the host bloodstream by ticks (Kjemtrup & Conrad, 2000), and IgG antibodies can block *B. microti* sporozoites from invading erythrocytes shortly following infection. If this

fails, NK cells and macrophages act to limit the extent of parasitaemia, (Homer *et al.*, 2000). Ultimately, a resolution stage begins, with parasitaemia levels peaking and then rapidly declining due to the action of CD4+ T cells and IFN-γ (Homer *et al.*, 2000; Igarashi *et al.*, 1999). While IgG is primarily involved in the early stages of infection, it is expected that circulating levels remain high following clearance, and as such are a useful bystander measure of immunocompetence (Barnard *et al.*, 1996b). IgG is also of relevance to this study because IgG can pass both transplacentally and through the milk in mammals (Diaz *et al.*, 2004; Field, 2005; Hanson *et al.*, 2003). Therefore the elevated levels seen in the offspring of *Babesia* treatment dams were to be expected.

Babesia treatment dams also gained more weight over pregnancy, lost more weight at parturition, and suckled their offspring more of the time. This may reflect an increased investment in the current litter, to the potential detriment of future reproduction. Iteroparus species, such as mice, usually do not put all of their investment in one reproductive bout, because it may reduce future reproductive success and therefore overall lifetime fecundity (Adamo, 1999; Williams, 1966). However, if longevity is reduced by a parasite, such a shift in investment priorities may occur (Javois & Tammaru, 2004). Bacteria infected crickets lay more eggs than uninfected controls, increasing present reproductive output when the chances of future output are lowered (Adamo, 1999). Immunologically challenged common eiders (*Somateria mollissima*) incubate eggs for longer, lose more weight in the process, and display higher levels of parental care after hatching (Hanssen, 2006) – a very similar pattern to that seen here. Nematode infected pregnant mice will produce

significantly and substantially larger litters (Kristan, 2004), but subsequent litters are smaller (Kristan, 2002a). However, this interpretation is somewhat weakened by the finding that *Babesia* treatment offspring gained less weight than control offspring during the pre-weaning period. The increased suckling seen may in fact have been driven by the offspring rather than the dams, and reflect the need to increase suckling time due to limited or lower quality milk production caused by post-infection reduced resources or reduced reproductive investment (Agrawal *et al.*, 2001).

Alternatively, or additionally, the patterns of maternal investment seen here may reflect immunocompetence-directed investment. Dams may be specifically priming offspring for life in an environment in which the risk of infection is high. Barnard *et al.* (1998) found that suckling behaviour affected both social rank and immunity in mice, with aggressiveness inversely proportional to maternal attention (but, as here, it is very hard to determine whether this was offspring or parent driven). The mice in Barnard's study which suckled more actually gained less weight – suggesting that increased suckling time does not reflect increased milk consumption. They then went on to become lower ranking, but with the associated benefits in immunocompetence (Barnard *et al.*, 1998). The same relationship between suckling and weight gain was found in previous studies on mice (Mendl & Paul, 1990). In this experiment, *Babesia* treatment sons also suckled more, gained less weight, and then went on to respond differently to both social and immunological challenges.

As adults, offspring which had developed in *Babesia* treatment dams showed a different hormonal response to control males when face with a novel social

environment. They reduced testosterone production on encountering non-sibling males for the first time, while control treatment males increased testosterone production. No effect of treatment on social behaviour was found. Evidence from other studies strongly implicates testosterone in the mediation of dominance-resistance trade-offs (Barnard *et al.*, 1993; 1994; Whitacre, 2001). In this study, the difference in hormonal response to social challenge was not reflected in recordable differences in aggression. This may have been due to the low levels of aggression naturally shown in BKW mice, and also to the fact that none of the males in this study had ever been singly housed prior to social grouping (single housing increases subsequent territorial behaviours (Van Loo *et al.*, 2003)). Despite the low levels of aggression recorded, it was possible to allocate ranks within each cage group, and higher-ranking males did show a downstream immunocompetence cost, exhibiting higher levels of infected cells at peak infection. This further supports the conclusion that a dominance-resistance trade-off was occurring.

Babesia treatment offspring subsequently showed a faster resolution phase in response to direct *Babesia* infection. Testosterone is known to interact with CD4+ T cells (Roberts *et al.*, 2001), which are important for the resolution phase of *B. microti* infection recovery (Homer *et al.*, 2000). The *Babesia* treatment offspring lost less weight over infection, indicating lower adverse effects from parasitism (Persing & Conrad, 1995). It is difficult to interpret the difference in IgG production over infection. It may be that in *Babesia* treatment offspring other immune cells and molecules, such as CD4+ T cells, are less diminished by the action of testosterone, and therefore the *Babesia* treatment offspring are less dependent on escalating

production of IgG. Alternatively, the difference may simply reflect a faster return to normal levels of immune components in the *Babesia* treatment males, as they clear the infection more efficiently.

While the findings here do not perfectly mimic those found following an indirect challenge by co-housing pregnant dams with infected neighbours, consistent trends emerge. In both cases maternal exposure leads to physiological changes in the dam, which ultimately filter down, affecting offspring response to social challenge (either behaviourally or physiologically) and to disease. In the presence of *Babesia*, offspring down-regulate investment in dominance acquisition, which in natural systems would lead to decreased access to mating opportunities (Barnard *et al.*, 1994; Meagher *et al.*, 2000; Waterman, 2007). In doing so, however, the offspring avoid the associated reduced resistance to disease (Barnard *et al.*, 1993; 1994; Whitacre, 2001). In an environment in which the risk of parasitism is high, cues experienced by the mother here lead to increased resistance in offspring. The results of this experiment emphasise the roles of both maternal effects and parasites, and the interplay between the two, in shaping life histories and disease susceptibility.

Chapter 6: Social challenge

6.1 Introduction

6.1.1 Dominance hierarchies

Social groups of animals are often characterised by dominance hierarchies, involving sustained aggressive/submissive competitive relationships among individuals (Barnard, 2004). In many cases, these stable hierarchies are constructed via pair-wise interactions, with each individual assessing of the likelihood of "winning" an encounter, based on their previous experience or cues relating to their own and their opponent's competitive ability (Dugatkin, 2002). Once an assessment is made, an individual may decide to persist in an interaction or withdraw accordingly, avoiding the costs of escalating aggression (Creel, 2001). As the dominant individual usually

ends up winning the resource, many have questioned why so many individuals "put up" with being subordinate (Clutton-Brock, 1998; Clutton-Brock *et al.*, 1997; Koolhaas *et al.*, 1999). One view is that submissive subordinates are simply making the best of their lot as poor competitors, avoiding conflict and possibly adopting different strategies to maximise fitness (Barnard *et al.*, 1994; Meagher *et al.*, 2000; Whitaker & Fair, 2002). In fact, some argue that dominance and subordination are in fact equally successful strategies in the long term, but do it in different ways (discussed more fully below) (Barnard, 2004; Creel, 2001; Mendl & Deag, 1995). Alternatively, in systems in which rank is a function of size or age, subordinates in some systems may simply be waiting until they are large or old enough to compete, or until the dominant individual is no longer competitive (Clarke & Faulkes, 2001; Clutton-Brock *et al.*, 2006; Faulkes & Abbott, 1997).

Whether transient or established, enforced or strategic, the social rank of an individual has a substantial impact upon its social interactions, health, and access to resources and mates (Barnard, 2004; Barnard & Behnke, 2006; Coltman *et al.*, 1999; Creel, 2001; Dloniak *et al.*, 2006). As expected, therefore, studies in primates, ungulates, canines, felids, and viverrids have all shown a strong relationship between social rank and reproductive success (Badyaev & Vleck, 2007; Bercovitch *et al.*, 2000; Berger, 1983; Bertram, 1975; Boesch, 1997; Clutton-Brock *et al.*, 1984; Clutton-Brock *et al.*, 1998; Hamilton & Zuk, 1982; Russell *et al.*, 2003; Sheldon & West, 2004; Simpson & Simpson, 1982). Social interaction provides some of the most important selective pressure on individuals, and it is therefore imperative that individuals respond to this pressure in an adaptive fashion, maximising the benefits

and minimising the costs of their particular status (Dloniak *et al.*, 2006). Perhaps most famously, male mating strategies are adapted according to the social rank in which a male finds himself. Males of many species will adopt a direct approach to accessing females if they are dominant, while adopting a "sneaky" approach if they are subordinate (Barnard, 1984; Lucas *et al.*, 1996; Norman *et al.*, 1999; Stapley, 2008). Foraging behaviour has also been shown to vary with dominance (Barta & Giraldeau, 1998).

6.1.2 Maternal rank as a measure of maternal quality

Dominance status, or rank, has often been used as a measure of maternal quality in studies of reproductive investment (Alberts, 1994; Clutton-Brock *et al.*, 1984; Gomendio, 1990). Researchers have classically determined female rank from the outcomes of agonistic encounters in which a winner and loser are unambiguously identified (Alberts, 1994; Silk, 1988). These agonistic encounters vary from aggressive interactions, such as chasing or attacking (Gomendio, 1990), to more passive displacements (Pusey *et al.*, 1997) or standardised threats (McFarland Symington, 1987; Simpson *et al.*, 1981). Using maternal rank as a measure of maternal quality, predictions can be made about litter size, interbirth interval, sex ratio and offspring quality (Barnard *et al.*, 1998; Bertram, 1975; Cameron & Linklater, 2000; Perret & Colas, 1997; Silk, 1988). For example, the Trivers-Willard hypothesis (Trivers & Willard, 1973) predicts that in polygynous species, dominant females would have more sons, which should be advantaged most in later life by their mother's high quality (see Chapter 3 Introduction). Such predictions are most

likely to be realised in populations with a clear and stable female dominance hierarchy, such as those of red deer (*Cervus elaphus*), chimpanzees (*Pan troglodytes*) and hyenas (*Crocuta crocuta*) (Boesch, 1997; Clutton-Brock *et al.*, 1984; Dloniak *et al.*, 2006). In rodents, however, female dominance is not well understood, and evidence for relationships between female rank and reproductive behaviour is limited.

6.1.3 Rank in rodents

Dominance hierarchies in male rodents are well described for many species (Waterman, 2007). In wild populations dominance is usually expressed as territoriality or defence of females. For example, resident yellow-bellied marmots (*Marmota flaviventrus*) have never been seen to lose an encounter with an intruding male (Goossens *et al.*, 1998). Male rodents will also directly defend females, and here more transient dominance leads to more escalated fighting (e.g. California ground squirrels, *Spermophilus beecheyi*, (Boellstorff *et al.*, 1994)). In captive populations of many species, territory and mate defence can translate into sustained aggressive/submissive dyads, as subordinate individuals are not able to avoid encounters through escape (Barnard *et al.*, 1994; Creel, 2001). Dominance dyads seen in the laboratory may not be representative of completely natural constructs, but they still usefully reflect differences in competitive ability, and have downstream consequences in a range of traits. Studies in laboratory rats (*Rattus norvegicus*), gerbils (*Meriones unguiculatus*), hamsters (*Mesocricetus auratus*) and mice have shown intricate interactions between male social rank, immunity, stress, longevity,

attractiveness and reproductive behaviour. On the whole, dominant male rodents are more attractive to females, less stressed, less immunocompetent and more reproductively successful than subordinates (Barnard *et al.*, 1993; 1996b; Clutton-Brock, 1998; Creel, 2001; Soloman & Keane, 2007).

Far less is known about the impact of rank on life history in female rodents (with the exception of communally breeding marmots and mole-rats; for a review see Faulkes and Abbott, 1997). In a recent review of sex ratio skewing in rodents, not one of the 23 studies discussed included maternal rank as a measure of maternal quality (Sikes, 2007). However, it is accepted that in wild populations females do express varying levels of territoriality, generally related to defence of pups from infanticide by male and female conspecifics (Faulkes & Abbott, 1997; Madden, 1974; Maestripieri, 1992; Schulte-Hostedde, 2007; Wolff, 1993; Wolff & Peterson, 1998). Dominance dyads have also been seen in female hamsters (Huck *et al.*, 1988a). Since male mouse territoriality in the wild translates into aggressive dominance dyads in captivity, it is reasonable to assume that such natural behaviour in female mice will translate in a similar way.

6.1.4 A social challenge experiment

In this experiment, I co-housed pairs of female laboratory mice, and examined social interactions in an attempt to ascertain whether or not female mice would readily form dominance dyads in a laboratory setting. I was interested in how this social

experience affected reproduction, and so following this social pairing I mated the females and examined various measures of reproductive investment, such a litter size, sex ratio, and maternal behaviour.

As well as not considering maternal rank when investigating parental investment in rodents, many studies examine litter size and sex ratio without considering other elements and consequences of maternal investment (Sikes, 2007). For example, when examining differential maternal investment in one sex over the other, differences in pre- and post-partum maternal expenditure in each sex (which may or may not ultimately lead to differences in sex ratio at age of independence) are frequently overlooked (Bercovitch *et al.*, 2000; Brown, 2001; Hewison & Gaillard, 1999; Sikes, 2007). In turn, the long term consequences for offspring fitness and life histories are often overlooked, with experiments failing to follow offspring into adulthood (but see Barnard *et al.,*1998; Drickamer *et al.*, 2000; Maccoll & Hatchwell 2003). In this study, I marked male and female pups pre-weaning, so that maternal interactions with each sex could be assessed. I also followed offspring into adulthood, and measured attractiveness (by mate choice trial with unrelated conspecifics), competitive ability (by co-housing in unrelated pairs) and nest-building ability in an attempt to gauge adult offspring quality.

Dominant females in other mammal species have been found to become sexually mature younger, produce more, higher quality young, and nurture young more effectively (Barnard, 2004; Clarke & Faulkes, 2001; McCann, 1982; Perret & Colas, 1997). There is also a relationship between dominance and sex ratio in many

polygynous species, with dominant individuals producing more sons (Barnard, 2004; Boesch, 1997; Byers, 1997; Cassinello, 1996; Clutton-Brock *et al.*, 1984). The differences between dominant and subordinate female reproduction can be attributed to social factors, such as dominant females experiencing better access to resources and reduced harassment (Appleby, 1980; Kojola, 1997; Pusey *et al.*, 1997). There are also hormonal causes and consequences of female dominance, which interact with reproductive schedules (Birkhead & Moeller, 1993; Dloniak *et al.*, 2006; Helle *et al.*, 2008; Sikes, 2007). If dominance dyads did emerge between female mice in this experiment (and these had similar social or hormonal consequences to those found in other species), I expected dominant individuals to become pregnant more easily, and have larger, more male-biased litters. I also expected dominants to spend more time nurturing offspring (possibly in a sex-biased fashion) and ultimately to produce young which were of a higher quality as adults.

6.2 Methods

6.2.1 Social pairing of dams

The subjects were 84 mice of the CD1 strain. This number comprised 42 subject dams (six weeks old) and 42 sire males (nine weeks old). Subjects were kept in standard cages and conditions throughout. Dams were allowed to settle in individual cages for one week, before being randomly paired and placed into fresh clean cages. Each dam was marked with an individually distinctive pattern using black eyelash dye. Continuous behavioural observations totalling 200 minutes (20 minutes per day, comprising four scans of five minutes each) per pair were carried out over the following ten days. Various social interactions were recorded in order to determine social rank within these pairs (detailed in Table 2.2 of the General Methods).

6.2.2 Mating phase

After the social pairing phase, dam pairs were separated and each dam placed into the cage of a randomly selected individually housed male. They were kept in these pairs for ten days, to allow dams to have a second oestrus cycle if they did not become pregnant in the first, maximising the likelihood of pregnancy and therefore minimising the risk of reduced sample sizes. After this period the males were removed and the dams housed singly until parturition.

6.2.3 Pre-weaning phase

Following parturition, litter size, pup weight and changes in dam weight were monitored until the pups were 11 days of age. At 11 days of age litters were sexed,

and all the pups of one sex were marked on the back using black eyelash dye. The sex marked was chosen randomly for each litter, and subsequent to marking was not known by observers. Six or seven scans were carried out per litter per day until weaning at 23 days of age (totalling 80 scans per litter). During scans the number of marked and unmarked pups involved in various behaviours were recorded, as well as the dam's behaviour (See General Methods Table 2.1). At weaning litters were standardised to four male and four female offspring (where possible) and re-housed in single sex fraternal or sororal groups of four. This was done to make following stages of the experiment manageable and due to the constraints of space and time.

6.2.4 Mate choice trials

At 45 days of age, offspring were involved in a mate choice trial. This involved placing non-sibling same-sex pairs of individuals in opposite corners of one side of a mate choice arena (Figure 6.1). For simplicity, the mate choice trial is here described for a female choosing between two males, but the same process was conducted for each male choosing between two females. Two unrelated males were placed in the small corner compartments of the arena. A female non-sibling was placed in the remaining half of the arena, and was able to make physical contact with both males through the bars (Figure 6.1). Behaviour of the female mouse was recorded for a five minute period to ascertain its mate preference (Table 6.1). Behaviours towards each male were scored depending on which corner of the cage the female was in at the time. Behaviours conducted in the central third of the female half were not recorded. In addition, total time spent opposite each male by the female was recorded.

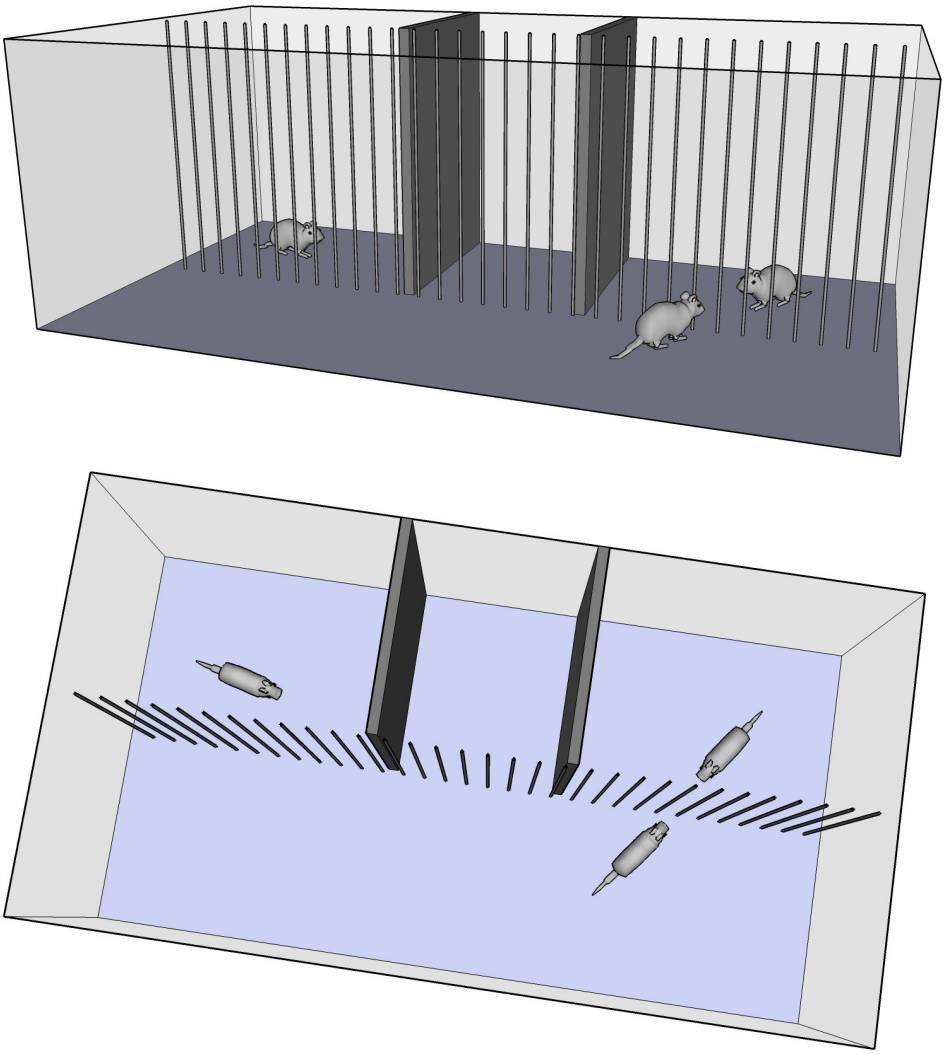


Figure 6.1: Mate choice arena. Front view (top) and aerial view (below). When male attractiveness was being assessed, males were placed in the small corner compartments. The female was separated from males by bars, but could move freely across her entire half of the arena. Males were unable to see each other, separated by two opaque plastic dividers. The whole arena was enclosed in a glass tank. When female attractiveness was being assessed, the whole arrangement was reversed, with two females placed in the small corner compartments and a choosing male placed in the undivided half.

Preference was determined by using the total number of interactive behaviours and the time spent near each male to create a principal component score (or preference

score) for each male in each trial. All males from subordinate mothers were paired with a male from a dominant mother. Each of these competitor pairs underwent two separate mate choice trials with two different females on different days. Attractiveness was calculated as the sum of the preference scores from the two trials. The whole process was repeated with male offspring choosing between female offspring to assess female attractiveness, and was scored in the same way. The mate choice arena was washed out with water and mild detergent between each trial to remove any residual odours.

Table 6.1: Behaviours recorded in the mate choice trial. Here shown as female offspring choosing male offspring, but the same behaviours were recorded when males were the choosers.

Behaviours	
Bars	Female touches bars
Climbing	Female climbs on bars
Glass	Female touches glass
Grooming	Female grooms self
Allogrooming	Female grooms male through the bars
Face-sniff	Female touches male face with own face
Body-sniff	Female touches male body with own face
Anogenital-sniff	Female touches male anogenital area with own face

6.2.5 Offspring social pairing

At 60 days of age, three quarters of all offspring (188 individuals) were housed with a novel non-sibling same-sex individual (different from those in the mate-choice trial). These social pairs were co-housed for five days, and a total of 30 minutes continuous behavioural observations were carried out per pair. Various social interactions were recorded in order to determine social rank within these pairs

(detailed in General Methods Table 2.2). The remaining third (71 individuals) were not paired due to the constraints of time and animal house space.

6.2.6 Nest building

Dam nests were scored based on the system explained in the General Methods. This was done at four points during the experiment:

1. During the settling period, before the social pairing of dams.
2. Following pairing, prior to mating.
3. During pregnancy, following removal of the sire male.
4. One day post-weaning, following removal of pups.

In addition to scoring dam nests, all offspring were housed singly at the end of the experiment, and a single nest building attempt was scored two days after being cleaned out.

6.3 Results

6.3.1 Maternal dominance dyads

Dams showed significantly different levels of aggression in novel social pairs (Chi-Squared tests for aggression in each pair, see Table 6.2).

Table 6.2: The majority of dam pairs showed significantly different amounts of aggression.

Pair	Aggressive acts by A	Aggressive acts by B	X^2	*Degrees of Freedom*	*P - value*
1	8	38	19.57	1	< 0.0001*
2	12	22	2.94	1	0.0863
3	4	12	4	1	0.0455*
4	10	17	1.81	1	0.1779
5	19	39	6.9	1	0.0086*
6	25	22	0.19	1	0.6617
7	28	18	2.17	1	0.1404
8	18	8	3.85	1	0.0499*
9	16	9	1.96	1	0.1615
10	20	29	1.65	1	0.1985
11	28	6	14.24	1	0.0002*
12	36	45	1	1	0.3173
13	16	40	10.29	1	0.0013*
14	25	10	6.43	1	0.0112*
15	8	12	0.80	1	0.3711
16	9	10	0.05	1	0.8185
17	36	14	9.68	1	0.0019*
18	16	3	8.89	1	0.0029*
19	20	12	2	1	0.1573
20	41	23	5.06	1	0.0244*
21	50	3	41.68	1	< 0.0001*
22	8	18	3.85	1	0.0499*

* Statistically significant at the 0.05 level.

It was therefore possible to divide each pair into a dominant and a subordinate individual, with dominants showing higher levels of aggression (Figure 6.2A, GLM: $F_{1,40} = 21.243$, $P < 0.0001$) and subordinates displaying higher levels of defensive

behaviour (Figure 6.2B, GLM: $F_{1,40} = 6.368$, $P = 0.016$). Dam social rank was not predictable by weight at pairing (GLM: $F_{1,40} = 0.064$, $P = 0.802$).

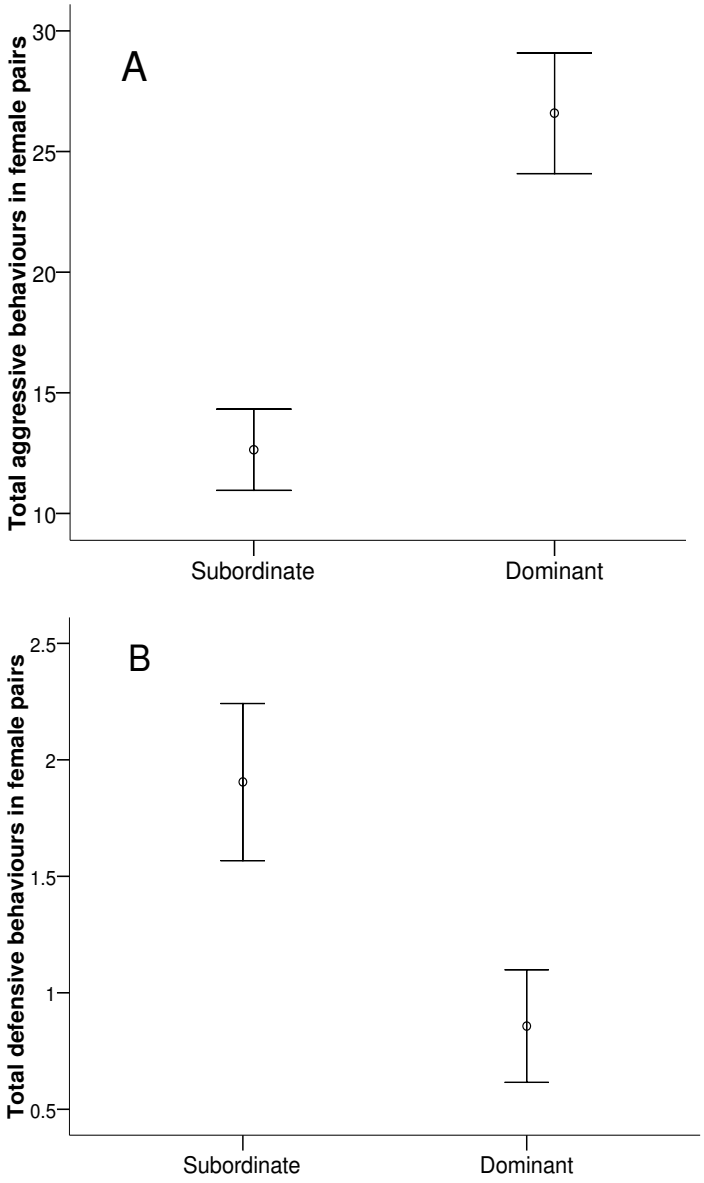


Figure 6.2: Aggressive (A) and defensive (B) behaviour in dams. Total acts observed during continuous focal scans. Error bars represent ± 1 standard error.

6.3.2 Pregnancy and litter composition

Subordinate dams were significantly more likely to become pregnant than dominant dams (Chi-Squared test: $X^2 = 4.286$, $df = 1$, $P = 0.038$). Twenty out of 21 subordinate dams became pregnant during the ten day mating period, compared to 15 out of 21 dominant dams. Dominant dams which did become pregnant gained more weight over pregnancy, and lost more weight at parturition, than subordinates (Figure 6.3: Repeated measures GLM: $F_{2,64} = 3.782$, $P = 0.028$).

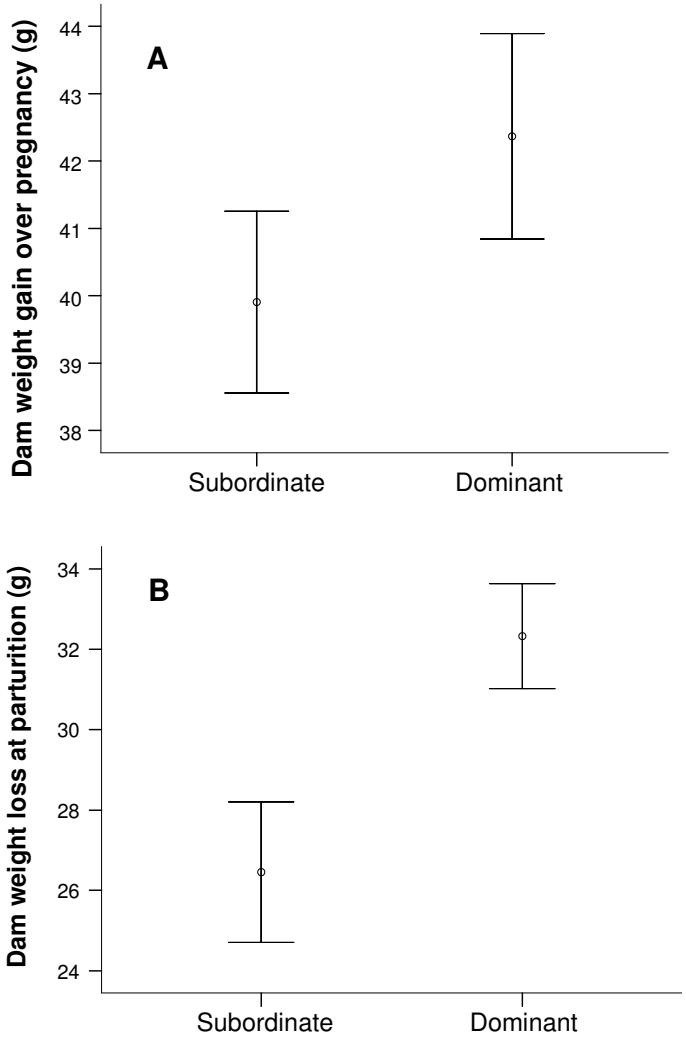


Figure 6.3: Dam weight changes over pregnancy. A. shows weight gain over pregnancy, B. shows weight loss at parturition. Error bars represent ± 1 standard error.

Dominant and subordinate dams did not produce significantly different sized litters (GLM: $F_{1,32} = 1.9$, $P = 0.178$). Dominant females produced litters which were on average 44% male, compared to subordinate litters which were 53% male, but this difference was not significant (GLM: $F_{1,31} = 2.325$, $P = 0.137$).

6.3.3 Maternal behaviour

Subordinate dams spent significantly more time suckling pups than dominant dams (Figure 6.4A: GLM: $F_{1,30} = 5.279$, $P = 0.029$). Dominant dams spent significantly more time allogrooming pups, though allogrooming levels were on the whole very low (Figure 6.4B: GLM: $F_{1,30} = 6.696$, $P = 0.015$). Overall, subordinate dams spent slightly, but not significantly, more time in contact with their offspring (Figure 6.4C: GLM: $F_{1,30} = 3.899$, $P = 0.058$).

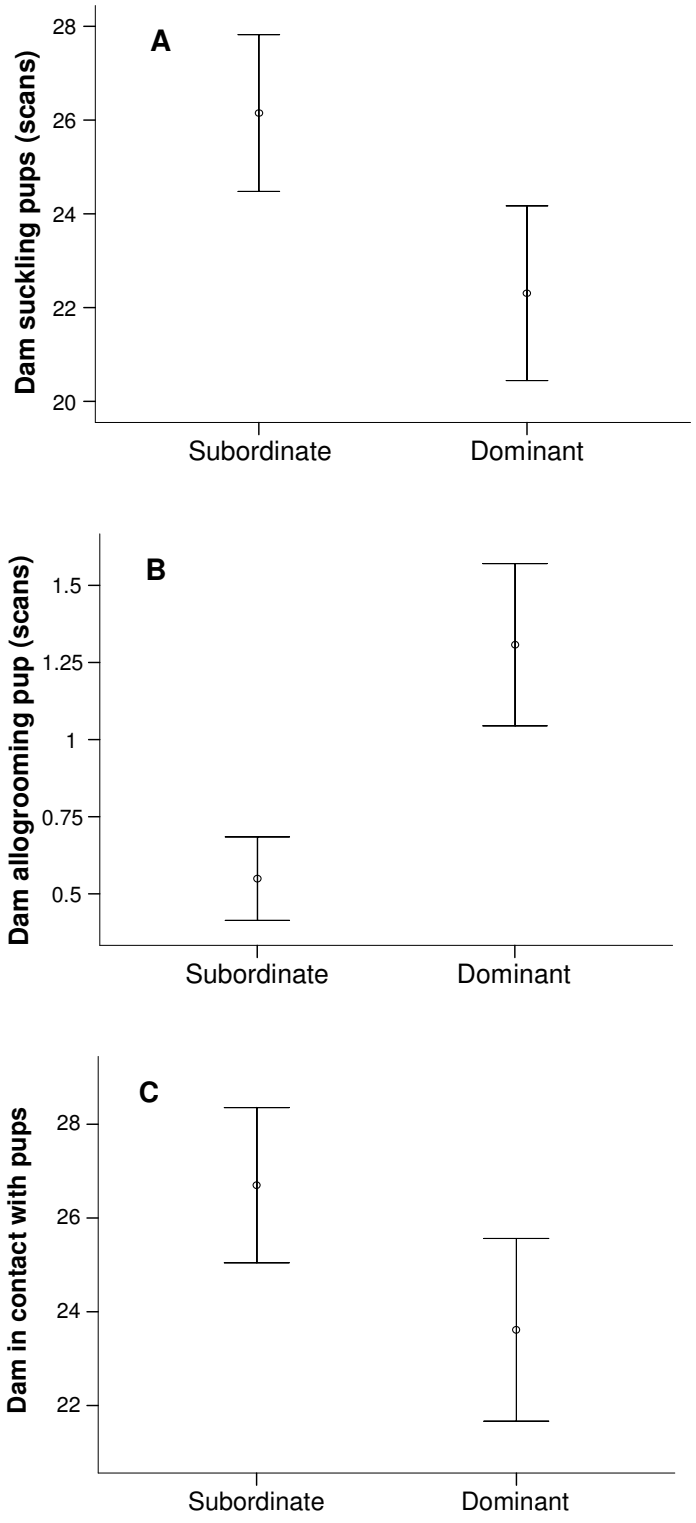


Figure 6.4: Differences between dominant and subordinate maternal behaviour. Behaviour totals calculated as total number of instantaneous focal scans in which dam displayed behaviour. Error bars represent ± 1 standard error.

There was no effect of social rank on dam feeding or grooming behaviour during the pre-weaning phase, or on pup loss post-partum (Table 6.3). There were also no interactions found between dominance and preferential suckling of sons or daughters (Table 6.3).

Table 6.3: No significant differences were found between dominant and subordinate dams in the following measurements.

	F	*Degrees of Freedom*	*P - value*
Dam grooming self[a]	0.009	1, 30	0.926
Dam feeding[a]	0.450	1, 30	0.507
Pup loss post-partum[a]	0.439	1, 31	0.513
Proportion of time sons spend suckling[b, a]	0.120	1, 31	0.731
Proportion of time daughters spend suckling[c, a]	0.255	1, 31	0.617
Difference between sons and daughters suckling[d, a]	0.652	1, 31	0.425

[a] GLM
[b] Calculated as the proportion of individual male pups scans spent suckling
[c] Calculated as the proportion of individual female pups scans spent suckling
[d] Calculated as proportion of male pup scans spent suckling minus the proportion of female pup scans spent suckling

6.3.4 Adult offspring

There was no effect of maternal dominance on adult offspring body weight for sons (Repeated measures GLM: $F_{3,384} = 0.954$, $P = 0.415$) or daughters (Repeated measures GLM: $F_{3,378} = 0.315$, $P = 0.815$). In novel social pairs, the offspring of dominant mothers were significantly more aggressive than the offspring of subordinate mothers (Figure 6.5: GLM: $F_{1,37} = 4.846$, $P = 0.034$).

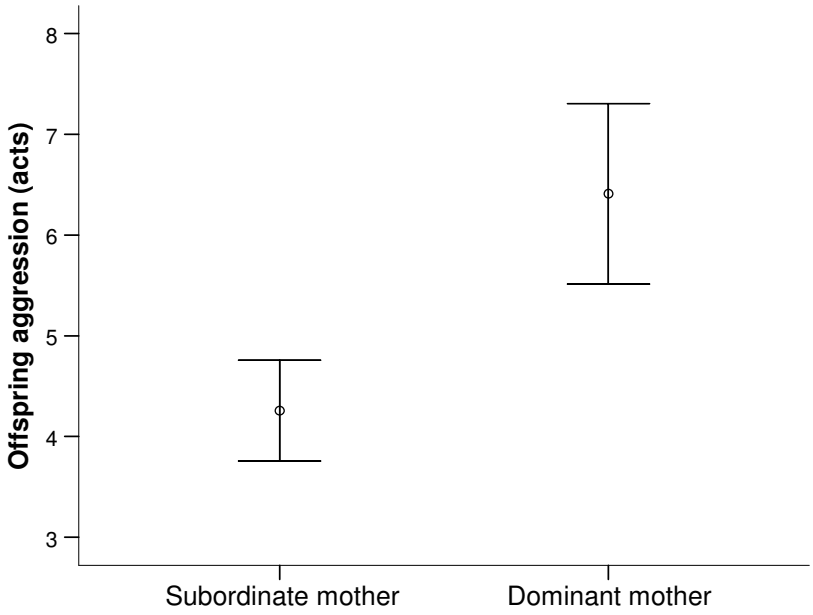


Figure 6.5: Differences in the aggressive behaviour of adult offspring. The graph shows the total number of instantaneous focal scans in which adult offspring displayed aggressive behaviours in novel social pairs. Error bars represent ± 1 standard error.

The difference in aggression did not translate into a significant difference in adult offspring social rank (Table 6.4). No difference was found in either male or female attractiveness, or nest building ability, as adults (Table 6.4). At autopsy, sons of dominant mothers were found to have significantly smaller testes than sons of subordinate mothers (dominant mean for combined testes weight = 0.283g, subordinate mean = 0.300g; GLM: $F_{1,32} = 4.685$, $P = 0.038$). There was no other difference in organ weight (Table 6.4).

Table 6.4: No significant differences were found between the offspring of dominant and subordinate dams in the following measurements.

	F	*Degrees of Freedom*	*P - value*
Son attractiveness [a]	0.366	1, 35	0.549
Son dominance [b]	0.063	1, 35	0.804
Son nest-building score	0.187	1, 35	0.668
Daughter attractiveness [a]	0.129	1, 31	0.072
Daughter dominance [b]	1.437	1, 31	0.239
Daughter nest-building score	0.411	1, 36	0.526
Son spleen	2.093	1, 32	0.158
Son heart	1.758	1, 32	0.194
Son thymus	0.917	1, 32	0.345
Son seminiferous tubule	0.450	1, 32	0.507
Son paired kidneys	0.596	1, 32	0.446
Son paired adrenal glands	0.547	1, 32	0.465
Son paired preputial glands	0.241	1, 32	0.627
Daughter spleen	1.731	1, 30	0.198
Daughter heart	0.952	1, 30	0.337
Daughter thymus	0.133	1, 30	0.718
Daughter paired kidneys	0.309	1, 30	0.582
Daughter paired adrenal glands	1.414	1, 30	0.244

All differences were tested using GLM. All organs analysed with terminal body weight as a covariate.

[a] Determined by the combined scores of two separate mate choice trials using non-siblings of the opposite sex. Dominant offspring always competing with subordinate offspring.

[b] Dominance ranks allocated based on behaviours observed during a six day social trial with novel individual of the same sex. All dominant offspring paired with a subordinate offspring.

6.4 Discussion

Significant differences in aggressive behaviour made it possible to assign dominance ranks in paired CD1 female mice. Differently ranked individuals went on to differ in their likelihood of becoming pregnant, with dominant females being less likely to conceive during a limited mating phase. Dominant dams which did become pregnant gained more weight over pregnancy, and lost more weight during parturition. Dominant mothers appeared to invest less in offspring post-partum, spending less time suckling offspring than subordinate mothers. The offspring of dominant mothers went on to behave differently as adults, displaying higher levels of aggression than those born to subordinate females.

The differences in conception rate may reflect differences in immediate investment in reproduction. Dominant individuals are known to invest heavily in maintaining dominance or territory ownership (Frank *et al.*, 1995; Mendl & Deag, 1995), and may do so to the detriment of immediate reproductive output. Such a trade-off might be mediated by the differences in hormone levels that have been associated with female social rank in many taxonomic groups. Circulating levels of androgens, estrogens and glucocorticoids are all affected by female rank, and in turn, affect reproductive physiology and behaviour (De Kloet *et al.*, 1999; Dloniak *et al.*, 2006; Sapolsky *et al.*, 2000). For example, circulating androgens in a dominant female rodents may act both to suppress ovulation (Sikes, 2007) and provoke aggression when a male is introduced, lessening the likelihood of lordosis behaviour (Birkhead & Moeller, 1993).

Dominant females which did become pregnant gained more weight during pregnancy and lost more at parturition than subordinate females (Figure 6.3). This might reflect increased investment in the litter, though this was not supported by post-partum behaviour. Dominant dams actually spent less time suckling their offspring than subordinates. Given this, it is perhaps not surprising that offspring from dominant mothers went on to become more aggressive adults, as maternal attention and aggression are known to be inversely correlated (Barnard *et al.,* 1998; see also Chapter 5 Results and Discussion). Offspring aggression may have alternatively or additionally been affected by hormonal levels in dams (a maternal effect) (Anway & Skinner, 2006); or the results may reflect a heritable component of aggressive behaviour (Foerster *et al.*, 2007).

This study shows that it is possible to determine rank in captive female mice, and that social experience has downstream effects on reproductive behaviour and offspring development. However, many of the predictions which might be made given the clear dominance dyads were not borne out. Dominant females in other species are known to become sexually mature younger, produce more young and nurture young more effectively, all of which leads to a positive reproductive skew in the direction of dominants (Barnard, 2004; Clarke & Faulkes, 2001; McCann, 1982). This was not found to be the case in this study, where in fact subordinates showed higher levels of fertility and maternal care. Similarly, the Trivers-Willard theory would have predicted higher sex ratios in dominant litters (see Chapter 3), or increased maternal investment in sons in these groups, but no such bias was found.

Indeed, if anything, the opposite skew was seen (though this was not significant), as seen in Chapter 3. There appeared to be no relationship between maternal dominance and offspring quality. None of the three measures used here to determine offspring quality – dominance, attractiveness, and nest building (linked to reproductive success in mice (Bult & Lynch, 1997)) – were found to be affected by maternal rank.

A limitation of this study arises from the assessment of female rank based on the artificial construct of aggression displayed in a captive environment (Creel, 2001). The conditions experienced here, even if they adequately provoked a demonstration of differences in competitive ability, may have failed to enable or initiate the appropriate coping mechanisms for those differences. In the wild, dominant females may have exclusive access to resources which would have advantaged them in this reproductive bout, whereas here all females were fed *ad libitum* and had protected nesting sites once pregnant. Such important differences in resource holding may initiate a cascade of strategic decisions which might result in the differences in sex ratio, gender-biased maternal investment, and adult offspring quality predicted by theory and previous studies. Additionally, in the wild subordinates are able to escape aggression, and dominants successfully exclude challengers, so both rank classes in this study may have experienced unnaturally high levels of stress. Stress hormones interact substantially with reproductive processes, and have long term sex-specific effects on offspring (Casolini *et al.,* 1997; Edwards & Burnham, 2001; Gluckman *et al.,* 2005; see also Chapter 4 discussion). In this study, these effects may have masked more subtle adaptive responses. Finally, if social aggression among females in the wild is not as intense as that seen here, mice may not have evolved the

appropriate behaviour modulating trade-offs. Broadly speaking, the selection pressures which might generate adaptive changes in reproductive output may be absent in the wild.

Despite the limitations of a laboratory study, this experiment has shown that female mice demonstrate measurable differences in aggressive behaviour, and will form dominance dyads in captivity. It has also shown that maternal dominance interacts with reproductive behaviour and offspring development, opening up avenues of investigation which might be most successfully explored in semi-natural enclosures which facilitate natural responses to dominance inequalities.

Chapter 7: Mate quality challenge

7.1 Introduction

7.1.1 Female mate choice

Female mate choice is widely accepted as a powerful mechanism driving the evolution of male traits through the process of sexual selection (Andersson & Simmons, 2006; Darwin, 1871; Williams, 1966). Rather than a passive acceptance of the winners of male-male contests, female mate choice has been shown to be an active and complex process (Craig & Morris, 2003; Soloman & Keane, 2007; Zala *et al.*, 2008a).

Firstly, it is now understood that female mate choice occurs at various stages in the reproductive process, and is often cryptic (Eberhard, 1996). As well as choosing

mating partners prior to copulation, females also select for paternity during copulation, post-copulation, and post-fertilisation (Birkhead & Moeller, 1993). During copulation a female can affect the likelihood of fertilisation by failure to transport sperm from the mate or failure to ovulate (Birkhead, 1998; Eberhard, 1996; Fedina & Lewis, 2007; McClintock *et al.*, 1982). Post copulation, but prior to fertilisation, females may eject sperm from certain individuals or enable sperm competition through multiple matings (Dean *et al.*, 2006; Jennions & Petrie, 2000; Pizzari & Birkhead, 2002; Pizzari & Birkhead, 2000). Post-fertilisation females may prevent implantation of conceptuses or terminate pregnancy (Berger, 1983; Bruce, 1959; De Catanzaro *et al.*, 1999; Kumar & Dominic, 1993; McClintock *et al.*, 1982; Wersinger *et al.*, 2008).

Secondly, cues used by females to determine mate quality are complex and multimodal. Females frequently use visual cues, such as elaborate sexual ornaments, colouration and symmetry (Godin & McDonough, 2003; Hamilton & Zuk, 1982; Ryan, 1998; Swaddle & Cuthill, 1994). Often information garnered from visual cues may be augmented by assessment of male displays or vocalisations (Candolin, 2003). Auditory and odour assessment is common in rodents (Guo & Holy, 2007; Soloman & Keane, 2007) and may yield information about traits as diverse as spatial ability (Spritzer *et al.*, 2005), disease status and resistance (Penn & Potts, 1998a; Zala *et al.*, 2008a; Zala *et al.*, 2004) and competitive ability (Zhao *et al.*, 2003).

Finally, not all females within a population prefer the same males. Individual preferences and experiences mean that two females will often select different mates.

In various experiments female rodents have been shown to prefer familiar males (Shapiro *et al.*, 1986), unrelated males (Barnard & Fitzsimons, 1988; Meagher *et al.*, 2000; Penn, 2002) and those of an opposite strain (Yanai & McClearn, 1973). Perhaps most significantly, females appear to select for genetic compatibility, and there is now a wealth of evidence showing that females across vertebrate taxa choose males with different immunocompetence genes (e.g. MHC) to their own (Craig & Morris, 2003; Jordan & Bruford, 1998; Milinski *et al.*, 2005; Penn, 2002; Penn & Potts, 1998b; Yamazaki *et al.*, 1976).

7.1.2 Mechanisms behind mate choice evolution

The complexity inherent in female mate choice mirrors the complexity that is evident in the mechanistic basis of the evolution of sexually selected traits. The offspring of choosey females may benefit from their mothers selectivity in a number of different ways. The male traits used by females to choose mates may reflect the potential for direct benefits for the offspring, such as access to territory or food, paternal care or protection (*Direct benefits hypothesis*) (Andersson & Simmons, 2006; Moller & Jennions, 2001). Certain male traits may have evolved due to pre-existing sensory biases in females. Females may have originally attended to certain cues for reasons unrelated to mate choice (e.g. foraging behaviour indicating food, or alarm calls indicating predators). Such attention biases could then have been exploited by males and ritualised into courtship displays which vary depending on male quality (*Sensory bias hypothesis*) (Andersson & Simmons, 2006; Barnard, 2004; Ryan, 1998). If females prefer a male trait (perhaps arbitrarily) which is heritable, their offspring

may inherit alleles both for the trait and the preference. If the offspring are successful reproducers, these alleles will spread in the population due to a self-reinforcing selective advantage, with females expressing the preference and males expressing the trait (*Fisherian runaway hypothesis*) (Fisher, 1930; Kokko, 2001).

Finally, indirect genetic benefits of female mate choice may come in the form of genetic complementarity. Evolving parasites and pathogens provide a dynamic selective landscape upon which host traits evolve (Drickamer *et al.*, 2000; Ilmonen *et al.*, 2007), and sexual selection occurs in this landscape. The ability of offspring to respond to a dynamic disease threat may be one of most important qualities determining viability and future reproductive success. Therefore a female can greatly advantage offspring by choosing males which maximise offspring genetic diversity, creating a "moving target" against rapidly evolving parasites (Penn & Potts, 1999). They can achieve this either by multi-mating (and thus producing more diverse litters) or by choosing males with different MHC alleles to their own (increasing individual heterogeneity of offspring (but see Ilmonen *et al.* 2007)). MHC-disassortative mating preferences additionally ensures females avoid mating with relatives, and thus avoid the associated fitness consequences of inbreeding (*Genetic compatibility hypothesis*) (Ilmonen *et al.*, 2008; Jordan & Bruford, 1998; McClelland *et al.*, 2003; Meagher *et al.*, 2000; Milinski *et al.*, 2005; Penn *et al.*, 2002; Potts *et al.*, 1991).

7.1.3 Male dominance

Despite the complexity, inconsistency, and individually varied nature of mate choice, certain male traits emerge as consistently preferred. Numerous studies have shown that females prefer unparasitised over parasitised males (Ehman & Scott, 2002; Kavaliers & Colwell, 1995; Kavaliers *et al.*, 2003; Penn & Potts, 1998a; Penn *et al.*, 1998; Zala *et al.*, 2004). Similarly, females in a number of species prefer unmated to sexually experienced males (Dale & Slagsvold, 1996; Huck *et al.*, 1986; Krames & Mastromatteo, 1973; Pierce & Dewsbury, 1991). Finally, male dominance is often reported to be a preferred trait (Havlicek *et al.*, 2005; Meagher *et al.*, 2000; Soloman & Keane, 2007; Waterman, 2007; Zhao *et al.*, 2003).

It has generally been assumed that the winners of male-male contests (i.e. dominant individuals, discussed more fully in the introduction to Chapter 6) are of better quality, and therefore it is directly and indirectly advantageous for females to mate with them. A dominant mate might bring to a female a territory, protection from infanticide, and access to food, as well as the genes which enabled the acquisition of dominance (Horne & Ylonen, 1998; Meagher *et al.*, 2000). However, few studies have actually examined the fitness consequences of mating with dominant males for the next generation (Cotar *et al.*, 2008; Qvarnström & Forsgren, 1998), and there is evidence for immediate fitness costs. For example, dominant males can experience sperm depletion due to high mating rate, and this may reduce the likelihood of successful fertilisation (Dewsbury, 1982; Pitnick & Markow, 1994; Sato & Goshima, 2007). Aggressive dominant males are also know to display reduced parental care in

some species (Forsgren, 1997; Wong, 2004). For example, male sand gobys (*Pomatoschistus minutus*) which win male-male competitions are less attentive when caring for eggs. As a consequence, egg hatching success is reduced, and in fact females are shown to prefer competition losers (Forsgren, 1997).

Most recently, investigation of sexually-antagonistic selection suggests that male dominance may have detrimental effects on female offspring fitness (Chippindale *et al.*, 2001; Foerster *et al.*, 2007; Turelli & Barton, 2004). As males and females have different roles in reproduction, selection drives them to different phenotypic optima (Rice & Chippindale, 2001). Sex-limited gene expression reduces this antagonism somewhat, but most genes are not sex limited, leaving substantial potential for conflict (Chippindale *et al.*, 2001; Morrow *et al.*, 2008). Empirical evidence for sexually antagonist selection is beginning to grow: dominant male red deer have daughters of low fitness (Foerster *et al.*, 2007); and mating success of male crickets (*Allonemobius socius*) is positively correlated with sons' mating success, but negatively correlated with daughters' reproductive success (Fedorka & Mousseau, 2004).

It is therefore not always a good strategy for females to select the most competitively able males (Qvarnström & Forsgren, 1998). In natural systems, high frequencies of paternity by dominant males may in fact not be a product of female choice, but rather an inevitability of male-male competition leading to differential access to mates. As Wong (2004) puts it, "the two processes of sexual selection are often confounded in time and space". Indeed, the two processes may even at times create opposing

selective pressures (Ulrika, 2004). Figure 7.1 details how these two elements of sexual selection might interact to determine male mating success (Qvarnström & Forsgren, 1998).

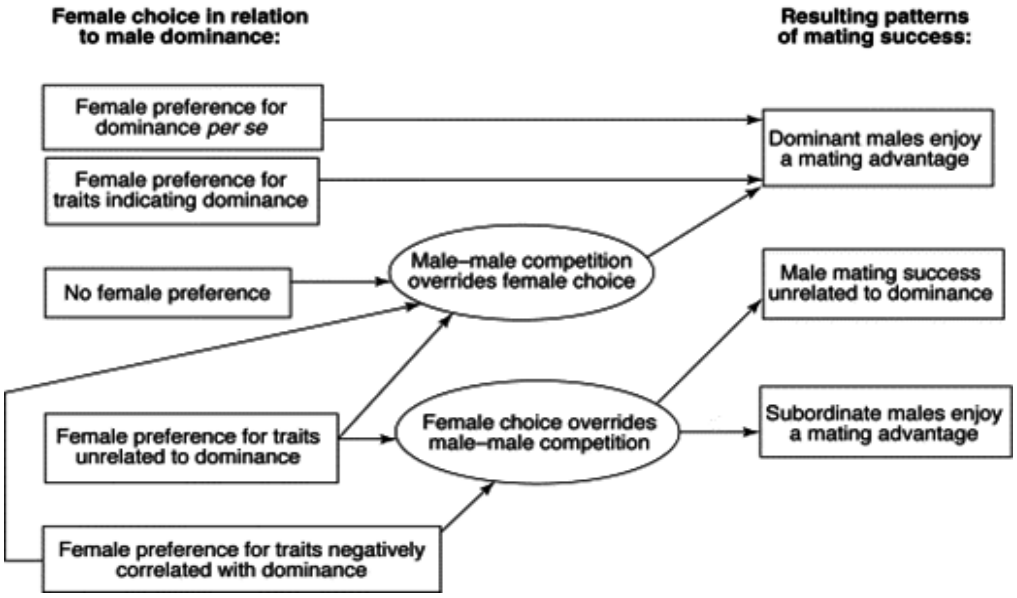


Figure 7.1: Female choice and male-male competition interact to determine male mating success. From Qvarnström & Forsgren, 1998.

7.1.4 A mate quality challenge experiment

Here, I examined the interaction between female preference and male dominance in mice. Within the controlled parameters of a laboratory environment, I tested whether or not females could determine future dominance of unfamiliar males (if there were cues of "dominance potential"). I also examined how female preferences changed with the acquisition of information and with familiarity, by co-housing females with male pairs, and then re-testing preferences. Ultimately, I assessed the long term fitness consequences of sire dominance and female preference, by examining

offspring attractiveness (through mate choice trials), dominance (through social pairing) and survival ability (through assessing nest building).

If male dominance is a sexually attractive trait in mice, as suggested by much of the literature (Soloman & Keane, 2007), I expected female mice to be attracted to dominant males. If dominance is also predictable, then this preference might be detectable before females had been co-housed with males, and then probably strengthened by the observation of dominant behaviour by those males when placed in dyads. However, if mating with dominants is costly, (and high dominant paternity levels seen in natural environments are in fact due largely to male-male competition) I expected females to prefer subordinates. Once mated, I expected reproductive output (such as likelihood of becoming pregnant and litter size) to reflect preferences. If dominance is determined by sex-limited or non-sexually antagonistic genes, the dominance of all offspring as adults would be likely to increase with male dominance. However, if sexually antagonistic genes were involved, then I would expect son quality to correlate positively with paternal quality, but daughter quality to correlate negatively. Offspring attractiveness was expected to follow a similar pattern.

Regardless of dominance, I predicted that preferred mates would lead females to have larger litters and higher quality offspring, based on a previous study in which female mice were allowed to express an "uninformed" preference (there was no period of observation) and then mated with preferred or non-preferred males (Drickamer *et al.*, 2000).

7.2 Methods

7.2.1 Mice and housing

The subjects were 120 mice of the CD1 strain. This number comprised 40 subject dams (six weeks old) and 80 sire males (nine weeks old). Subjects were kept in standard cages except during the observation phase of the experiment when sire male pairs and subject females each had one half of a large divided cage (see Chapter 4, Figure 4.2).

7.2.2 First mate choice trial

Dams were allowed to settle in individual cages for one week, while sire males were kept socially housed, before being randomly paired with a new male and placed into opposite corners of the mate choice arena (see Chapter 6, Figure 6.1). No visual or tactile contact was possible between the males and they were unable to leave their allocated corner. Dams were placed in the remaining half of the arena, and were able to make physical contact with both males through the bars. Behaviour of the dam was recorded for a five minute period to ascertain preferences (see Chapter 6, Table 6.1 for details of behaviours). Preference was determined by using the total number of interactive behaviours and the time spent near each male to create a principal component score (or preference score) for each male in each trial. The male with the higher preference score from each trial was considered the preferred male. The mate choice arena was washed out with water and mild detergent between each trial to remove any residual odours.

7.2.3 Observation phase

Following the first mate choice trial, dams were placed into one side of a divided cage (Chapter 4, Figure 4.2). The two males from the mate choice trial were housed in the other half of the divided cage, separated from the dam by a clear perforated Perspex partition. Continuous behavioural observations totalling 60 minutes (10 mins per day) per pair were carried out over the following six days. Various social interactions between the males were recorded, in order to determine social rank within these pairs (See General Methods Table 2.2 for details). In all cases a dominant and a subordinate male could be identified (see Results). In addition to behavioural observations, coat condition was scored on a scale of one to ten for each sire male (see General Methods, Figure 2.1).

7.2.4 Second mate choice trial

The observation phase was followed by a second mate choice trial, which followed the same procedures as the first and involved the same three individuals in the same mate choice arena compartments.

7.2.5 Mating phase

After the second mate choice trial, dams were randomly allocated one of the two males, and re-housed with this male in a new clean standard cage. Allocation ensured that there were even numbers of dams paired with subordinate and dominant males. They were kept in these pairs for ten days (this meant that dams would have two oestrus cycles if they did not become pregnant in the first, maximising the likelihood

of pregnancy and therefore minimising the risk of reduced sample sizes), after which the males were removed and dams housed singly until parturition.

7.2.6 Pre-weaning phase

Following parturition, litter size, pup weight and changes in dam weight were monitored until the pups were 11 days of age. At 11 days of age litters were sexed, and all pups of one sex were marked on the back using black eyelash dye. The sex marked was chosen randomly for each litter, and subsequent to marking was not known by observers. Six or seven scans were carried out per litter per day until weaning at 23 days of age (totalling 80 scans per litter). During scans the number of marked and unmarked pups involved in various behaviours were recorded, as well as the dam's behaviour (detailed in General Methods Table 2.1). At weaning, litters were standardised to four male and four female offspring (where possible) and re-housed in single-sex fraternal or sororal groups of four. This was done to make following stages of the experiment manageable and due to the constraints of space and time. One hundred and ninety offspring were used in following stages of the experiment.

7.2.7 Offspring mate choice trials

At 45 days of age, offspring were involved in a mate choice trial. This involved the same mate choice arena and protocol as detailed in Chapter 6 (and see Figure 6.1). All offspring from subordinate sires were randomly paired against an offspring from a dominant sire. Each of these competitor pairs underwent two separate mate choice

trials with two different randomly selected non-sibling opposite-sex choosers on different days. Attractiveness was calculated as the sum of the preference scores from the two trials.

7.2.8 Offspring social pairing

At 60 days of age, offspring were housed with a randomly chosen novel non-sibling same-sex individual (different from those in the mate-choice trial). These social pairs were co-housed for five days, and a total of 30 minutes continuous behavioural observations were carried out per pair. Various social interactions were recorded, in order to determine social rank within these pairs (detailed in General Methods Table 2.2).

7.2.9 Nest building

Dam nests were scored at four points during the experiment based on the system described in the General Methods.

1. During the settling period, before the observation phase.
2. Following the observation phase, prior to mating.
3. During pregnancy, following removal of the sire male.
4. One day post-weaning, following removal of pups.

In addition to scoring dam nests, all offspring were housed singly at the end of the experiment, and a single nest building attempt was scored two days after being cleaned out.

7.2.10 A note on sample sizes and animal welfare

This experiment was preceded by a pilot study designed to test the cage design and mouse behaviour during the observation phase. Three divided cages were set up for a week long trial. Due to problems with suppliers BKW mice were used in this pilot, and only low levels of aggression were recorded. Six days appeared to be a good length of time in which to determine male dominance.

In the experiment proper, CD1 mice were used, for reasons explained in the General Methods (Section 2.1). CD1 males exhibited much higher levels of aggression in this set-up. As a consequence, 11 of the original 41 pairs were separated very early because of concern for the welfare of the subordinate males, and 11 dams were accordingly removed from the experiment. This led to lower sample sizes than had originally been planned and may have had consequences for the statistical power of the study. With hindsight, it can be seen that the ideal experimental design would pair CD1 males in the presence of a female for only one or two days, to protect subordinate males from the most aggressive males without affecting the experimental power.

7.3 Results

7.3.1 Sire males

Sire males showed significantly different levels of aggression in the social pairs co-housed with dams in divided cages (Chi-squared tests for aggression in each pair, see Table 7.1). It was therefore possible to divide each pair into a dominant and a subordinate individual.

In the mate choice trials conducted prior to the observation phase, dams showed a significant preference for males which were to go on to become subordinate, preferring 19 out of 30 eventual subordinates, compared to 11 out of 30 dominants (Chi-Squared test: $X^2 = 4.267$, $df = 1$, $P = 0.039$). This was also shown as a preference for males which later displayed significantly less aggression than non-preferred males (Figure 7.2A, GLM: $F_{1,58} = 7.284$, $P = 0.009$). Following the observation phase, the preference for subordinates was even more pronounced, with nine out of 30 dominants preferred, compared to 21 out of 30 subordinates (Chi-Squared test: $X^2 = 9.600$, $df = 1$, $P = 0.002$). Preferred males were accordingly less aggressive (Figure 7.2B, GLM: $F_{1,58} = 13.221$, $P = 0.001$). Being preferred before the observation phase did not affect the likelihood of a male being preferred afterwards (Chi-Squared test: $X^2 = 0.267$, $df = 1$, $P = 0.606$).

Table 7.1: The majority of sire pairs showed significantly different amounts of aggression. All males are shown here, including those separated part way through the observation phase.

Pair	Aggressive acts by A	Aggressive acts by B	X^2	*Degrees of Freedom*	*P - value*
1	2	0	0.50	1	0.4795
2	3	0	1.33	1	0.2482
3	0	12	12.00	1	0.0005*
4	11	0	11.00	1	0.0009*
5	12	7	1.32	1	0.2513
6	12	4	4.00	1	0.0455*
7	0	13	13.00	1	0.0003*
8	1	2	0.00	1	1.0000
9	0	6	4.17	1	0.0412*
10	0	2	0.50	1	0.4795
11	0	14	14.00	1	0.0002*
12	1	12	9.31	1	0.0023*
13	1	21	18.18	1	< 0.0001*
14	0	4	2.25	1	0.1336
15	0	26	26.00	1	< 0.0001*
16	6	0	4.17	1	0.0412*
17	0	10	10.00	1	0.0016*
18	4	0	2.25	1	0.1336
19	5	0	3.20	1	0.0736
20	19	1	16.20	1	0.0001*
21	5	5	0.00	1	1.000
22	10	0	10.00	1	0.0016*
23	2	6	1.13	1	0.2888
24	2	14	9.00	1	0.0027*
25	18	1	15.21	1	0.0001*
26	15	2	9.94	1	0.0016*
27	5	0	3.20	1	0.0736
28	1	6	2.29	1	0.1306
29	0	10	10.00	1	0.0016*
30	8	1	4.00	1	0.0455*
31	0	4	2.25	1	0.1336
32	0	26	26.00	1	< 0.0001*
33	14	1	11.27	1	0.0008*
34	9	3	3.00	1	0.0833
35	4	22	12.46	1	0.0004*
36	7	0	5.14	1	0.0233*
37	11	1	8.33	1	0.0039*
38	18	1	15.21	1	0.0001*
39	0	14	14.00	1	0.0002*
40	0	38	38.00	1	< 0.0001*
41	0	5	3.20	1	0.0736

* Statistically significant at the 0.05 level.

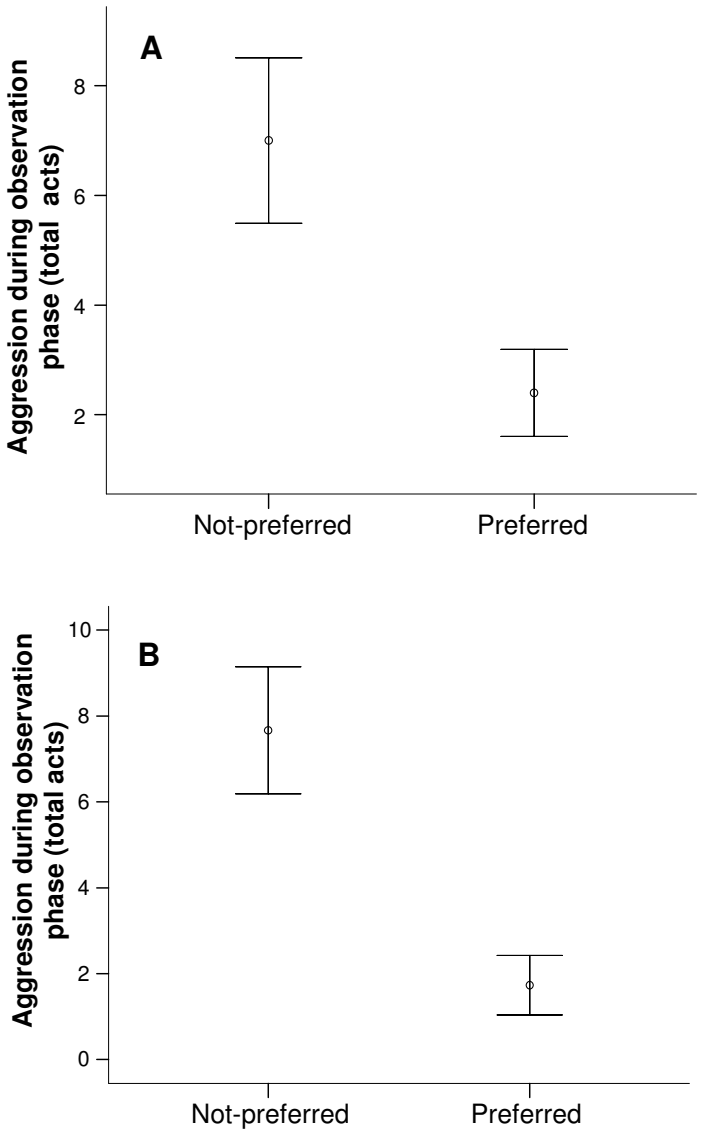


Figure 7.2: Relationship between dam preference and male aggression. Total attacks observed during the observation phase in relation to A: dam preference shown before, and B: dam preference shown after the observation phase. Error bars represent ± 1 standard error.

Following pairing for the observation period, dominant males had significantly better coat condition (Figure 7.3, GLM: $F_{1,58}$ = 12.436, P = 0.001). At autopsy, males

which had been dominant in the observation phase had significantly smaller kidneys and spleens than subordinates (Figures 7.4, Table 7.2). No other organs were affected by dominance (Table 7.2).

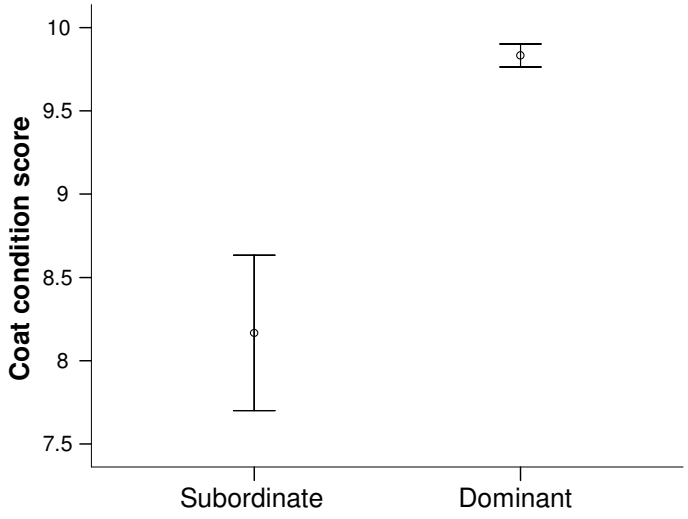


Figure 7.3: Relationship between dominance and coat condition. Male coats scored at the end of the observation phase. Error bars represent ± 1 standard error.

Table 7.2: Differences between dominant and subordinate male organ weights at autopsy. All analysed using GLM, with terminal body weight as a covariate.

	F	***Degrees of Freedom***	***P - value***
Thymus	0.952	1, 56	0.334
Heart	0.457	1, 56	0.502
Spleen	11.295	1, 56	0.001
Seminiferous tubule	3.770	1, 56	0.058
Paired kidneys	5.134	1, 56	0.028
Paired testes	3.555	1, 56	0.065
Paired adrenal glands	1.006	1, 56	0.320
Paired preputial glands	0.122	1, 56	0.728

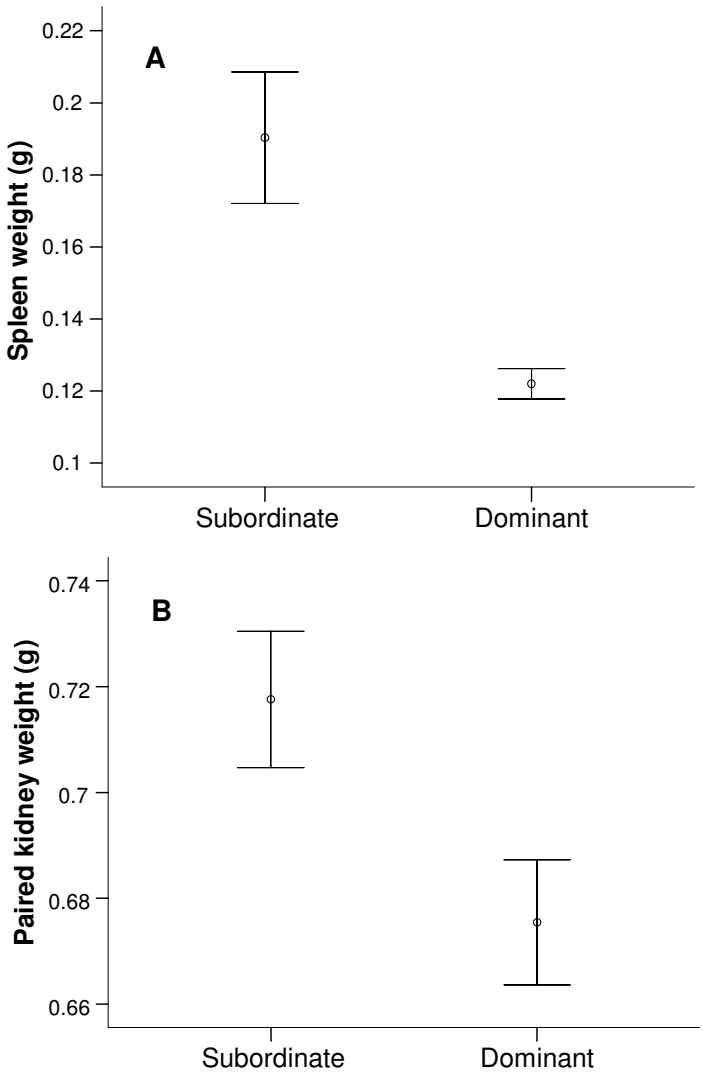


Figure 7.4: Relationship between sire male dominance and organ weights. A: spleen weight, and B: paired kidney weight. Error bars represent ± 1 standard error.

7.3.2 Maternal response to sire dominance

Dominance of the sire males did not affect the likelihood of dams becoming pregnant (Chi-Squared test: $X^2 = 1.154$, $df = 1$, $P = 0.283$), nor did dam preference for males before or after the observations period (Chi-Squared test: before: $X^2 = 0.084$, $df = 1$, $P = 0.773$; after: $X^2 = 1.489$, $n = 30$, $P = 0.222$). However, those which did become pregnant by dominant males had significantly larger litters than those which were pregnant by subordinates (Figure 7.5, GLM: $F_{1,24} = 4.299$, $P = 0.049$).

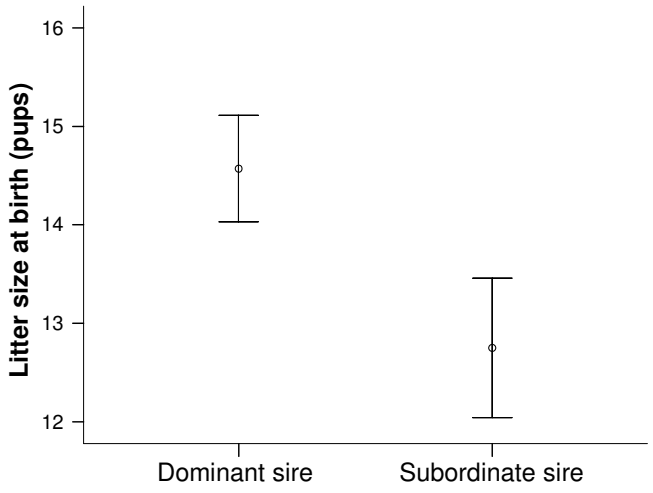


Figure 7.5: Relationship between sire male dominance and litter size at birth. Error bars represent ± 1 standard error.

There was no effect of sire dominance on pup loss post-partum (Repeated measures GLM with number of alive pups as response variable: $F_{4, 96} = 1.649$, $P = 0.168$) or litter sex ratio (GLM: $F_{1, 24} = 0.203$, $P = 0.657$). There were no effects of sire dominance on measures of maternal behaviour (Table 7.3).

Table 7.3: No significant differences were found between dams mated with dominant or subordinate sires in the following measurements.

	F	*Degrees of Freedom*	*P - value*
Dam suckling pups[a]	0.448	1, 22	0.448
Dam in contact with pups[a]	0.326	1, 22	0.574
Proportion of time sons spend suckling[b, a]	0.038	1, 23	0.846
Proportion of time daughters spend suckling[c, a]	0.062	1, 23	0.806
Difference between sons and daughters suckling[d, a]	0.110	1, 23	0.743
Pup weight at three days (not disturbed at birth) [a]	0.194	1, 23	0.664
Pup weight gain to weaning [e]	0.448	4, 92	0.412

[a] GLM
[b] Calculated as the proportion of individual male pups scans spent suckling
[c] Calculated as the proportion of individual female pups scans spent suckling
[d] Calculated as proportion of male pup scans spent suckling minus the proportion of female pup scans spent suckling
[e] Repeated measure GLM (with pup weight at each time point as response variable)

7.3.3 Adult offspring

Paternal dominance was not found to affect the future dominance of sons (Chi-Squared test: X^2 = 2.671, *df* = 1, *P* = 0.102) or daughters (Chi-Squared test: X^2 =0.268, *df* = 1, *P* = 0.605). There was also no effect on the attractiveness of either sex (GLM on combined mate choice trial preference scores – sons: $F_{1, 26}$ = 0.117, *P* = 0.735; daughters: $F_{1, 24}$ = 0.531, *P* = 0.473) or nest building ability (GLM – sons: $F_{1, 23}$ = 0.011, *P* = 0.917; daughters: $F_{1, 25}$ = 0.159, *P* = 0.693). Dam preference for the sire, taken as her preference in the second mate choice trail, was also not found to have an effect on any of these measures of offspring quality (Table 7.4).

Table 7.4: No significant differences were found between dams mated with preferred or non-preferred sires in the following measurements.

	Statistic	***Degrees of Freedom***	***P - value***
Son dominance[a]	X^2 = 0.677	97	0.411
Daughter dominance[a]	X^2 = 0.863	93	0.353
Son attractiveness[b]	*F* = 0.123	1, 26	0.729
Daughter attractiveness[b]	*F* = 0.538	1, 24	0.470
Son nest-building score[b]	*F* = 0.292	1, 23	0.594
Daughter nest-building score[b]	*F* = 0.602	1, 24	0.445

[a] Chi-squared test
[b] GLM

7.4 Discussion

In this study of the relationships between sire dominance, female preference and reproductive output, female mice were found to prefer subordinate males. This preference was expressed both before males had been socially challenged, and following a period of pairing which the dam was able to observe. Male mice readily formed dominance dyads with a significantly different level of aggression expressed by the dominant and the subordinate in the majority of pairs. Dams which were mated with dominant males had larger litters than those mated with subordinates, but there was no effect of female preference on reproductive output. Sire dominance was not found to affect any of the measures of offspring quality used.

At first encounter, dams were able to distinguish between male mice which would go on to become dominant and those which would not. It is possible that prior to the first mate choice trial, males had already acquired social status in their group housing. Those which were more aggressive are likely to have been dominant in these groups, and therefore have been expressing the associated behaviours, vocalisations and social odours (Barnard *et al.*, 1997b; Barnard *et al.*, 1996b; Barnard & Luo, 2002; Gourbal *et al.*, 2004). Aggressive individuals would also be more likely to be dominant in the new pairs, which may explain the predictive nature of the dam preference. The fact that initial preference did not predict later preference suggests that individuals themselves were not preferred. Following the observation period, "informed" dams continued to show a preference for subordinate individuals.

Female preference for subordinate individuals has been reported previously (Göransson *et al.*, 1990; Howard *et al.*, 1997; Ophir & Galef, 2003; Stapley, 2008) but is rare in rodents (Soloman & Keane, 2007). Ensminger and Meikle (2004) found that females mice spent more time near the odours of less aggressive males, and were more likely to produce litters if housed with less aggressive pairs of males. It is possible that dams are stressed by aggressive behaviour, and that while resources held by dominants might be attractive to females (Alatalo *et al.*, 1986; Jones, 1981; Oakeshott, 1974), aggressive behaviour itself is aversive, perhaps because it suggests an environment in which the risk of infanticide is high (Ebenspurger, 1998; Ensminger & Meikle, 2005; Haller *et al.*, 1998).

Previous studies have suggested that female house mice are actively polyandrous, and will mate multiply with both subordinate and dominant males (Busser *et al.*, 1974; Rolland *et al.*, 2003). This may be done to reduce infanticide (Ebenspurger, 1998), increase genetic heterogeneity of litters (Jordan & Bruford, 1998) or promote sperm competition (Birkhead, 1998). Indeed, approximately 23% of litters from natural populations of house mice show mixed paternity, and the level of multiple matings is estimated between 45% and 70%, indicating that sperm competition is common in this species (Dean *et al.*, 2006). Rolland and colleagues (2003) showed that female mice are indiscriminate with regards to dominance at the beginning of a multiple mating bout, but will usually end up with the dominant male. It is possible that the subordinate preference seen in this experiment would not have been borne out had females been able to go on and mate freely with their preferred male, nor might it have been reflected in the paternity of the offspring.

Dams mated with dominant males went on to produce larger litters than those mated with subordinates. This did not reflect many smaller pups, as the pups in dominant litters were not on average smaller than subordinate litter pups. The larger litters may reflect adaptive reproductive decision making by females, adjusting litter size in keeping with the likelihood of high resource holding by sire males. Differential allocation of reproductive investment based on mate quality is well established (Forkman & Corr, 1996; Sheldon, 2000; Thornhill, 1983), and would suggest that, while associative female preference measured in the mate choice trial was for subordinates, reproductive investment favoured dominant males.

Alternatively, the larger litters seen here may have been a result of male-mediated effects. Male mice respond to social circumstances in an adaptive fashion, maximising the benefits and minimising the costs of their particular status (Dloniak *et al.*, 2006). This includes adaptation of reproductive investment according to social rank (Barnard, 1984; Lucas *et al.*, 1996; Norman *et al.*, 1999). Dominance increases the likelihood of mating, and therefore dominant males increase investment in sperm production accordingly. Simultaneously, subordinates may direct resources elsewhere. In this experiment, pairing appeared to be costly to subordinate males, expressed as significantly poorer coat condition (which would reduce the thermoregulatory value of the coat, and increase the risk of parasitism and infection). The subordinate males also had enlarged kidneys and spleens. Kidney growth may reflect reduced inclination to urinate in (what is perceived to be) another's territory. Renal damage in subordinate captive mice has been reported previously (Nevison *et*

al., 2003). Spleen growth usually reflects immune challenge, and immunological responses here may have been provoked by a combination of poor coat condition and minor injuries from aggressive interactions. Subordinates may therefore have been directing resources into these immediate needs, rather than reproductive investment. In rodents, the probability of fertilization is increased by sperm volume, size and motility (Stockley & Preston, 2004; Waterman, 2007) and indeed social dominance is found to enhance sperm production and quality across taxa (Stockley & Purvis, 1993). In this experiment, sperm volume of dominants would not have been depleted by high mating rates as it might in natural environments (Dewsbury, 1982). Therefore, the patterns seen in this experiment are consistent with dominance-mediated differences in male reproductive investment.

Dominant males may also have been better able to manipulate maternal investment, to the detriment of the females' fitness but the benefit of their own. Conflict between the sexes is an increasingly accepted aspect of sexual reproduction, as the costs and benefits of reproduction are rarely equal for males and females (Parker, 2006; Penn & Smith, 2007; Tregenza *et al.*, 2006). Male mice do not usually benefit from the future reproduction of their current mate, and so will gain by ensuring that females invest the maximum amount in the current litter. Females, on the other hand, may increase their fitness by spreading their investment across multiple reproductive bouts. Thus a battle of the sexes emerges, which is usually mediated by sex specific gene expression and imprinting (both inter-locus and intra-locus conflict) (Parker, 2006; Tregenza *et al.*, 2006). It is likely that individuals differ in their competitive ability on this battle field, and the larger litters produced by dams which mated with

dominant sires may reflect a positive relationship between social dominance and sexual conflict dominance in male mice. Such a relationship would create an additional cost for females choosing to mate with dominant males, and may also help to explain the preference seen here.

No differences were found between the adult offspring of subordinate and dominant males in any of the traits measured. It is possible that no significant differences were found because of the low sample sizes (insufficient power) caused by necessary separation of a quarter of the sire pairs due to high levels of aggression (see methods). However, other significant effects were found despite the small sample, and it is possible that sire dominance does not have strong fitness consequences for offspring. Either way, it is not possible to draw conclusions from this experiment about the sex-linked or sexually antagonistic nature of any genes which may underlie dominance, or the downstream effects of possible changes in maternal investment due to mate rank. Following the offspring still further and examining their own reproductive output and behaviour might have offered such possibilities, and would be an exciting future direction for similar experiments.

Chapter 8: General Discussion

In this thesis I examined the flexibility of reproductive resource allocation by female mice in response to manipulation of their nutritional, immunological and social environment. I found that changes in the local environment and body condition of females impacted upon their reproductive behaviour, and in turn, upon the phenotypes of their offspring. In five separate experiments, I found that food restriction, direct and indirect exposure to disease, maternal social experience and mate quality all affected female reproductive output with life history consequences for offspring.

Alongside the more subtle interactions found between the environment and maternal investment, which are discussed in detail in the relevant chapters, there were important key findings. In response to a nutritional challenge, female mice produced more male offspring. Co-housing pregnant females with diseased conspecifics led to the production of offspring which were more resistant to disease and less aggressive as adults. Females that were directly infected with a parasite prior to pregnancy produced offspring that were more resistant to disease and showed abnormal hormonal response to socialisation. Dominant females were less likely to become pregnant than subordinates, suckled young less, and produced offspring that were more aggressive as adults. Females showed a preference for subordinate males, but produced larger litters if mated with a dominant. These key findings point to flexibility in life history allocation in mice. The flexibility shown has fitness

consequences for both females and their offspring, suggesting that there is adaptive transgenerational phenotypic plasticity in mice.

While interpreting the results of each experiment, I was continually faced with a key limitation. I hoped to understand better the flexibility of behaviours which had evolved in the natural environment, but was testing my hypotheses in the artificial setting of the laboratory. The laboratory facilitated careful experimental design in which a limited number of factors could be manipulated, and in which many subtle responses could be measured. However, it is important to acknowledge the potential problems which captivity creates. In artificial circumstances, subjects will still respond from a behavioural repertoire which has been naturally derived, and responses may or may not be appropriate or have the desired outcome (Barnard, 2007). This may in turn lead to stress and frustration, and the exhibition of additional inappropriate behaviours. This constraint applies to all five experiments, but may be particularly important when considering dominance relationships. Dominance dyads may clearly emerge in captive pairs, and probably reflect natural competitive ability, but aggressive interactions cannot result in natural outcomes. Subordinates in cages cannot flee aggressive dominants, and so may experience unusually high levels of stress which may affect behaviour and physiology in other areas. Similarly, captive dominants continually fail to expel "intruding" subordinates, and the frustration experienced may similarly lead to stress or inappropriate allocation of resources (Barnard, 2004; Creel, 2001). Given this, the observed effects of female rank in Chapter 6 and of male rank in Chapter 7 on female preferences and reproduction might not reflect natural responses to dominance interactions. These experiments

crucially demonstrate aggressive differences between individuals, and the potential impact of these differences on attractiveness and reproduction. However, to understand better the nature of the interactions between dominance and life history strategies, open field experiments enabling more natural interactions may be necessary.

The mice in these experiments consistently showed physiological and behavioural responses to changes in the social or immunological environment, even when those changes were indirect. This leads me to consider seriously the welfare implications of most laboratory animal housing arrangements, where olfactory and auditory information about individuals in other cages is constantly available to subjects. This information will regularly contain cues about conspecific disease, reproductive state and social status. As caged individuals are unable to respond to these cues in a natural way (i.e. by fleeing or seeking the source of the cue) they may experience high levels of frustration or stress (Olsson & Westlund, 2007). Individuals co-housed in aggressive pairs may similarly be exposed to stressors from which they cannot escape. Even if the welfare of the animals used is not considered to be a priority, the implications for scientific research in general are great. I have shown that *Mus musculus*, the species used most in medical research (ASPA, 2007), respond in complex, substantial, and unpredictable and ways to ambient cues. In circumstances where subtle physiological or behavioural reactions are of importance (e.g. pharmaceutical trials) failure to understand the complexity of natural mouse behaviour may lead to false and potentially dangerous conclusions.

Due to the constraints of the experimental process (e.g. limited time, space and money) I was sometimes prevented from following exciting new directions highlighted by practical experience and preliminary results. In future experiments I think it would be very interesting to examine the relationship between nest building and fertility in both male and female mice (a relationship suggested by the findings in Chapter 3). Similarly, it would be of great interest to know if female offspring responded in a similar way to the maternal immunological challenges described in Chapters 4 and 5. Future experiments to examine the nature of the immunological cues detected in Chapter 4, and the mechanisms underlying the transgenerational immune priming in both chapters, would also greatly add to the findings here.

Perhaps one of the most frustrating aspects of behavioural ecology is that, in the absence of long term evolutionary experiments, it is almost impossible to test rigorously the adaptive nature of behavioural responses. However, such testing is fundamental to our understanding of behaviour, and manipulative experiments on model organisms play a crucial role. Nonetheless, relating the findings of these experiments to real world scenarios greatly helps to determine whether the strategies adopted are indeed adaptive. For example, it would be extremely interesting to understand more clearly the nature of dyadic dominance relationships found in these experiments. I would like to know how accurately they reflect more complex hierarchies in free house mouse communities. It is important to understand if dominance is intrinsic and to some extent heritable, or if it is a transient construct of individual pair-wise interactions. Better understanding of this would greatly help in the interpretation of my findings.

In the general introduction, I introduced *Mus musculus* as an ideal species to use in the investigation of the interaction between environment and life history strategies due to their adaptability to their social, physical, nutritional and immunological surroundings. A corollary of this is that I have chosen to study flexibility in a particularly flexible organism. Given this, and the limitations of laboratory experiments, caution is required in drawing from my findings broad conclusions about the flexibility of life history strategies in general. However, there is no question that environmental challenges imposed on the mice in these experiments caused changes in their reproductive behaviour in a way which would have fitness consequences, and therefore would be likely to be acted upon by natural selection.

During my investigation of the literature, I found myself continually excited by the seemingly limitless theoretical discussions surrounding the concepts investigated here. Individuals evolve due to competing selective pressures in natural environments which are continually changing, and which contain similarly evolving biotic components. In such a dynamic adaptive landscape predictions about strategic resource allocation can become incredibly complicated. In reviewing sex ratio skewing (discussed in Chapter 3), I noticed that many of the most conclusive experiments were carried out in monotocous species. In polytocous species such as mice, there is an additional dimension to the reproductive trade-off. Even with a constant level of maternal quality or resource availability, multiple reproductive strategies might be optimal. The optimal size and number of offspring in a litter (e.g. many small or few large) should interact strongly with the sex ratio, making the

curve of the optimal reaction norm three-dimensional (Stearns & Koella, 1986). When environmental conditions change, females should alter their position on this three-dimensional surface in a way which will produce the greatest benefit from that environmental change.

The reaction-norm surface introduced above might help us to predict how individuals should respond, but in reality it is already a huge oversimplification. Real-world females must also battle the pressures of parasitic manipulation, sexual conflict, parent-offspring conflict and the resource requirements of future reproduction, and thus the surface begins to have infinite dimensions. Add to this the apparently predictive nature of phenotypic plasticity and the transgenerational consequences of many adaptive responses, and interpreting or predicting life history strategies becomes overwhelmingly complex. Rather than leaving us feeling defeated, this complexity should fuel our determination to better understand the evolutionary processes underlying life history strategies. Collaboration between the approaches of comparative analysis, manipulative experimentation, genetic investigation and theoretical modelling will without doubt be invaluable in this endeavour.

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Appendix – Publication

Publication from the findings of Chapter 5:

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Mothers produce less aggressive sons with altered immunity when there is a threat of disease during pregnancy

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Maternal experience before and during pregnancy is known to play a key role in offspring development. However, the influence of social cues about disease in the maternal environment has not been explored. We indirectly exposed pregnant mice to infected neighbours by housing them next to non-contagious conspecifics infected with *Babesia microti*. We examined the effect of this indirect immunological exposure on both the females and their adult offspring. Exposed females had higher levels of serum corticosterone and increased kidney growth compared with those with uninfected neighbours. These exposed females subsequently produced offspring that as adults showed an accelerated immune response to *B. microti* and less aggression in social groups. We suggest that ambient information regarding disease is used adaptively to maximize offspring survival and reproductive success in a challenging environment. Our results shed light on the impact of social information and maternal effects on life histories, and have important consequences for our understanding of epidemiology and individual disease susceptibility in humans and other animals. They also lead us to question the suitability of some laboratory housing conditions during experimental procedures, which may impact negatively upon both animal welfare and the validity of animal science.

Keywords: life history; host–parasite; maternal effect; disease susceptibility; trade-off

1. INTRODUCTION

Ambient immunological information, such as that detected from the odours and behaviour of diseased individuals, is increasingly understood to play a key role in both immunity and behaviour (Zinkernagel 2000; Kilpimaa *et al.* 2004; Brennan & Zufall 2006; Zala *et al.* 2008*b*). The presence of diseased individuals is shown to cause dramatic and wide-ranging behavioural and physiological responses in neighbouring conspecifics (Kavaliers *et al.* 2003; Gelperin 2008). For example, female mice show preferences for the odours of unparasitized males in mate choice experiments (Penn *et al.* 1998; Ehman & Scott 2002; Kavaliers *et al.* 2003), while healthy rats (*Rattus norvegicus*) mimic the organ growth and hormone production of immunologically challenged neighbours (Fernandes 2000). In humans, body odour has long been used by physicians to diagnose diseases such as typhoid (baked bread odour) and yellow fever (meaty odour) (Penn & Potts 1998). It is known that many species of animals, including ourselves, are able to detect disease status in others, particularly in social odours (Kavaliers *et al.* 2005; Zala *et al.* 2008*a*). It is not known, however, if perception of this information benefits the receiver in any way, how it is used by individuals to make assessments about the risk of current disease in the immediate environment, and how it is exploited subsequently to maximize reproductive success.

The study of the transgenerational induction of traits is also gaining prominence. This occurs when information in the environment of the mother causes a change in the phenotype of the offspring, and is commonly known as a maternal effect (Agrawal *et al.* 1999; Marshall & Uller 2007; Rickard & Lummaa 2007). It is accepted that certain cues in the maternal environment, e.g. the prevalence of predators, can lead to behavioural or morphological changes in the following generation (Agrawal *et al.* 1999). It is also accepted that direct maternal infection with pathogens can have immunological consequences for offspring (Grindstaff *et al.* 2006). The adaptive nature of these consequences is still being actively debated (Groothuis *et al.* 2005*a*,*b*; Marshall & Uller 2007).

Here, we combine these two growing fields of investigation to test the hypothesis that cues about the disease status of neighbours are exploited by female mice in reproductive decision making. Specifically, we tested the idea that ambient immunological information is used to gauge the threat of disease to self and offspring, and that the capacity to resist infection in offspring in subsequent adult life is adjusted accordingly.

We tested our hypothesis using BKW laboratory mice and the intraerythrocytic parasite *Babesia microti*, a well-established host–parasite system used in our laboratories

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† We would like to dedicate this paper to Chris Barnard, who sadly died in June 2007, aged 55. Chris provided the inspiration for this work, and continues to be an inspiration to us all. He is greatly missed.

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Figure 1. Partitioned cages used during stimulus phase. Dams were housed on one side of the divided cage, separated from stimulus males by a perforated Perspex partition.

(Barnard *et al.* 2005; Barnard & Behnke 2006). *Babesia* species are tick-borne haemoprotozoan parasites that infect virtually all mammalian species, with significant economic consequences in domestic animals and human health implications (Kjemtrup & Conrad 2000; Kim *et al.* 2007). In mice, *B. microti* induces high but transient parasitaemias, which are quickly cleared (clearance beginning approx. 10 days after infection; Homer *et al.* 2000). Antibodies can block *B. microti* sporozoites from invading erythrocytes shortly following infection. If this fails, natural killer cells and macrophages act to limit the extent of parasitaemia, by production of gamma-interferon (IFN-γ), tumour necrosis factor-alpha (TNF-α), nitrous oxide (NO) and reactive oxygen species (Homer *et al.* 2000). There is also suggested involvement of the recently discovered MetHb-pseudoperoxidase pathway (Bogdan 2007; Jiang *et al.* 2007). Ultimately, a resolution stage begins, with parasitaemia levels peaking and then rapidly declining due to the action of CD4+T cells and IFN-γ (Igarashi *et al.* 1999; Homer *et al.* 2000). Following primary infection, mice are protected against future infection by the action of CD4+T cells and IFN-γ, with little or no requirement for B-cells or antibodies (Igarashi *et al.* 1999).

Our experiment involved housing pre-mated dams, opposite groups of stimulus males (figure 1), which had either been infected with *B. microti*, or subject to one of four control treatments. We used partitioned cages that were designed such that the dams could receive auditory, visual and olfactory information from their neighbours, but had no direct contact with them beyond potentially touching whiskers. Direct transmission of *B. microti* to our dams could not occur as it is dependent on a tick vector (Randolph 1991), which was absent, or direct exchange of blood, which was not possible. To test our hypothesis, we examined the effect of experimental treatment on maternal physiology and behaviour, and then adult offspring social behaviour and immune response to disease challenge.

2. MATERIAL AND METHODS

(a) *Mice and housing*

The subjects were 300 mice of the BKW strain (supplied by B & K Universal Ltd, Hull, UK). This number comprised 50 subject dams (seven weeks old); 200 stimulus males (three to five weeks old); and 50 sire males (nine weeks old). We kept subjects in standard polypropylene cages ($48\times15\times13$ cm: model M3, North Kent Plastics, UK) except during the stimulus phase of the experiment when stimulus male groups and subject females each had one-half of a large divided cage (figure 1; $28\times45\times13$ cm: model MB1, adapted specifically for this experiment). All cages and cage sections contained wood shavings as a floor substrate, a cotton nestlet for bedding material and a cardboard tube. Subjects had ad libitum access to standard laboratory rodent food pellets and water. Room temperature was maintained between 20 and 22°C and humidity between 45 and 55 per cent. All animals were maintained under a 12 : 12 h reversed light : dark cycle with lights on at 20.00 h, and illuminated by a dim red light during the dark cycle to facilitate observations.

(b) *Mating*

We introduced dams to the home cages of randomly allocated single-housed sire males, and kept them in these pairs for 6 days. This method was used to minimize sire–dam aggression, and maximize likelihood of impregnation (Koyama 2004), while still leaving sufficient gestation time for the stimulus phase.

(c) *Stimulus phase*

Following the mating phase, we separated each dam from her sire and rehoused her in a divided cage. The divided cage also housed four stimulus males, from which she was separated by a clear perforated Perspex partition. Stimulus males had been equally, randomly divided into five treatment groups. Each group contained 40 subject males: (A) *Babesia treatment*: infection with 5×10^7 red blood cells harbouring *B. microti* (infection treatment); (B) *Sham Babesia*: sham infection of *B. microti*, comprising only the vehicle used to suspend erythrocytes in treatment A (infection vehicle control treatment); (C) *SRBC treatment*: inoculation with 5×10^7 sheep red blood cells (immune activity treatment); (D) *sham SRBC*: sham inoculation of SRBCs, comprising only the vehicle used to suspend the SRBCs (immune activity vehicle control treatment); (E) *complete control*: no treatment (unmanipulated control). Immediately following treatment, stimulus males were housed in within-treatment groups of four on one side the divided cages. Multiple stimulus males per dam were used to standardize the stimulus exposure to each dam (i.e. to minimize the effects of variance among males). Four stimulus males would allow for this standardization, without increasing the risk of inter-male aggression (high in groups of two or three) or causing overcrowding (Sherwin 2002; Van Loo *et al.* 2003). Subject dams were divided into one of five indirect exposure groups, with

10 females per group. We kept dams in this stimulus phase for 10 days. At the end of the stimulus phase, we transferred the dams to a new clean cage to give birth.

(d) *Pre-weaning phase*

At 11 days of age, all pups were sexed and each litter was reduced to four males (three males in the case of three litters that had only three males each). This was to enable manageable sample sizes for individual observations both pre-weaning and in subsequent phases. Male pups were chosen because previous work has shown important dominance–resistance trade-offs in male mice (Barnard *et al.* 1997*a*,*b*, 1998). A total of 145 male pups were included in the following stages of the experiment, from 37 females (13 dams did not become pregnant in the mating phase, but the likelihood of becoming pregnant was not affected by treatment (χ^2-test: $X^2=1.663$, $n=50$, $p=0.197$)). We marked the male pups in an individually distinctive pattern using black eyelash dye (Colorsport, Brodie and Stone Plc, London, UK). At 24 days of age, all mothers were removed from litters, and pups (subject males) were left in their fraternal groups until 50 days old.

(e) *Social grouping and single housing phases*

At 50 days of age, all subject males were separated from their siblings. Approximately two-thirds of the subject males (88 mice) were rehoused with three novel males from the same treatment group, with whom they were allowed to establish dominance hierarchies. Continuous behavioural observations totalling 175 min (10 or 15 min per day) per group were carried out over the following 16 days. All observations were carried out during the active dark cycle of the day, at regular intervals between 0800 and 1900 h. Various social interactions were recorded, including the number of attacks, mounts and aggressive allogrooms, in order to determine social rank within these groups. The remaining third (57 mice) were housed singly to act as a non-socialized treatment. No effect of socializing treatment on infection profile was found (repeated-measures general linear model, GLM: $F_{5,134}=0.612$, $p=0.691$; PC1 score GLM: $F_{1,143}=0.364$, $p=0.547$), so data from socially and singly housed males were pooled in subsequent analysis. At the end of this phase, all males were removed and housed singly in new clean cages.

(f) *Infection phase*

At 70 days of age all of the adult offspring, all now housed singly, were injected with 5×10^7 red blood cells infected with *B. microti*. The time course of the infection was closely monitored by taking blood samples every other day until clearance. No other infection or immune challenge was given to the adult offspring.

(g) *Technical procedures*

All inoculations, infections and sham manipulations involved a single intra-peritoneal injection of 200 µl Hanks' solution, containing the appropriate inoculants. Stimulus treatment group E received no injection. All monitoring, sampling and handling for stimulus treatments A and C were repeated accordingly in sham treatments B, D and E. Sham infection and inoculation involved the introduction of Hanks' solution only. All subject males were infected in a random order with 5×10^7 infected red blood cells of *Babesia*. The King's 67 strain of *B. microti* was used throughout, and frozen stock was first passaged five times in BKW mice before being used on stimulus or subject males.

To monitor *Babesia* infection, a peripheral tail vein was nicked and a single drop of blood was transferred to a glass microscope slide every other day during the infection. The drop was immediately smeared to give monolayer of erythrocytes, then fixed and stained. For staining, fixed slides were placed in a solution of one part Giemsa stain to three parts Sorenson's buffer for 40 min, before rinsing in Sorenson's buffer and drying in air. Larger blood samples (50 µl) were also collected in heparinized haematocrit tubes, on no more than three occasions per animal, and never twice within a two-week period. Dams were sampled at the start and end of the stimulus phase. Offspring had 50 µl blood samples taken at weaning, prior to and at the end of social grouping. An additional blood sample was taken from all animals during autopsy.

The larger blood samples were assayed for testosterone (males only), corticosterone and total IgG (used as a bystander measure of immunocompetence; Barnard *et al.* 1996) using kits or reagents supplied by IDS Ltd, Tyne and Wear, UK (testosterone); R & D Systems Europe Ltd, Abington, UK (corticosterone); and Universal Biological, Cambridge, UK (IgG). All plates were processed using Micro plate Manager v. 5.2. In all cases, blood samples were anonymized and analysed in a random order, so that it was not possible for investigators to know the treatment group or relatedness of individual samples. In a number of cases, limited serum volumes precluded reliable estimates for all three serum factors at all time points from certain individuals. As a consequence, sample sizes of some analyses vary.

Autopsies were conducted in random order on subject males between the ages of 90 and 100 days, and on dams following weaning, when dams were approximately 100 days old. Autopsy involved the removal and weighing of the thymus, heart, spleen, left and right kidneys, left and right adrenal glands (males and females), uterus (females), left and right testes, seminiferous tubules and left and right preputial glands (males).

(h) *Statistical analysis*

Analysis was carried out using SPSS 15 (SPSS Inc., Chicago, IL, USA). Alpha was set at 0.05 and all tests were two-tailed. All analyses were based on pooled control treatment groups B–E as no significant differences were found between these groups (see the electronic supplementary material). Where appropriate, values were nested within dam (which was included as a random factor), or averaged per dam to avoid within-litter pseudoreplication. Principal components analysis conducted on five correlated variables describing individual infection profiles generated a principal component (PC1) with an eigenvalue of 2.652. Individual scores for this first component were then used in further analysis. Where appropriate, body mass was fitted as a covariate. As mentioned above, limited serum volumes precluded reliable estimates of serum factors for certain individuals. As a consequence, sample sizes and degrees of freedom vary for some analyses. *A priori* power tests were conducted to determine minimum sample sizes necessary to detect treatment effects in order to minimize animal use.

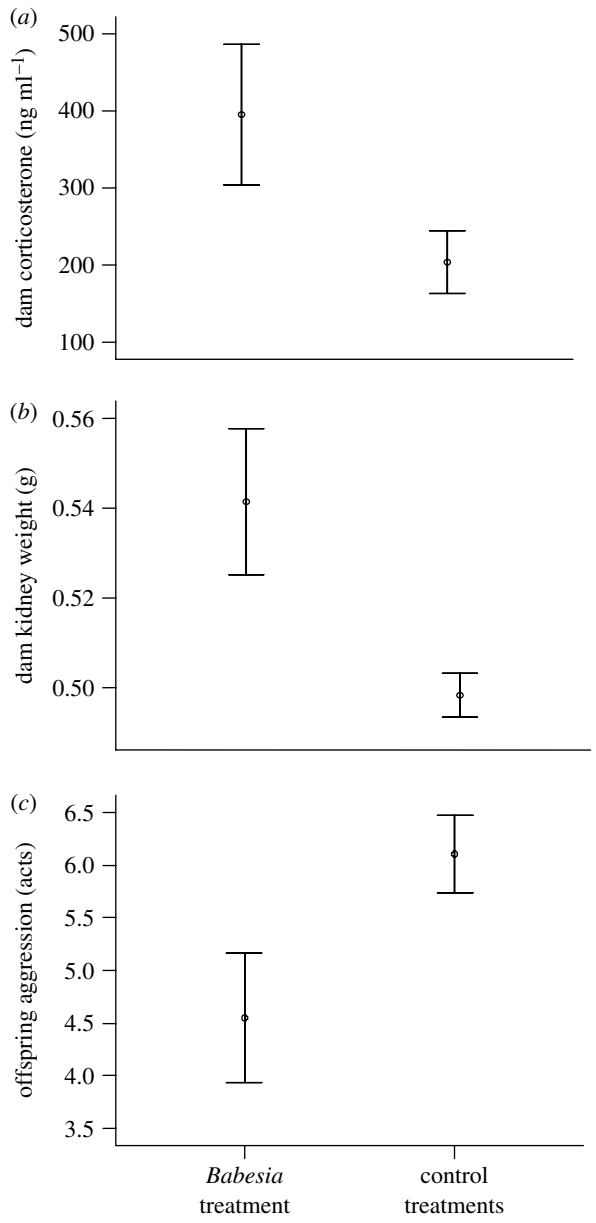

Figure 2. Treatment effects on dam physiology and offspring behaviour. Differences between dams that had shared partitioned cages with males infected with *B. microti* and those that shared with control males in (*a*) change in dam corticosterone across stimulus phase ($n=23$ dams), (*b*) weight of both the kidneys of dams at autopsy ($n=37$), and (*c*) adult offspring aggression, measured as the total observed acts in novel social groups ($n=88$ male offspring from 37 dams). Error bars represent ± 1 standard error (s.e.).

3. RESULTS

During the stimulus phase, we found that the pregnant dams housed opposite infected males (*Babesia* treatment) had blood serum levels of corticosterone twice the values recorded from dams housed opposite control males (absolute corticosterone following stimulus phase, GLM: $F_{1,20}=4.897$, $p=0.039$; change in corticosterone over the stimulus phase: figure 2*a*, GLM: $F_{1,20}=4.931$, $p=0.038$). Also, dams from the *Babesia* treatment had kidneys that were 8 per cent larger at autopsy than those of dams in control treatments (figure 2*b*, GLM: $F_{1,34}=9.391$, $p=0.004$). There was no effect of treatment on dam IgG (repeated-measures GLM: $F_{2,33}=0.819$, $p=0.494$) or spleen size (GLM: $F_{1,34}=1.537$, $p=0.224$).

Following the stimulus phase, all dams were transferred to new clean cages to give birth. There was no effect of treatment on litter size at birth ($F_{1,35}=0.612$, $p=0.439$) or litter sex ratio ($F_{1,35}=0.837$, $p=0.367$). At 11 days of age, all litters were standardized to four males. When re-housed with novel males as adults, offspring from *Babesia*-treatment dams were significantly less aggressive than those from dams in control treatments (figure 2*c*, GLM: $F_{1,42}=5.508$, $p=0.024$). While testosterone levels in offspring did predictably (Barnard *et al.* 1994) correlate with aggression (Pearson's correlation: $n=86$, $r=0.295$, $p=0.006$), experimental treatment was not found to have a significant effect on this hormone (repeated-measures GLM: $F_{3,33}=1.708$, $p=0.184$). There was no effect of treatment on offspring IgG (repeated-measures GLM: $F_{3,33}=1.021$, $p=0.397$).

Finally, all offspring were rehoused singly into new clean cages, and infected with *B. microti*. Offspring from *Babesia*-treatment dams showed a different response to disease across the period of infection (figure 3*a*; repeated-measures GLM: $F_{5,30}=2.612$, $p=0.045$). This difference reflected an accelerated time course of infection, with offspring from *Babesia*-treatment dams showing earlier onset, peak and clearance of infection than offspring from control dams (table 1; figure 3*b*, GLM: $F_{1,34}=4.314$, $p=0.045$).

4. DISCUSSION

We found that the pregnant dams housed in partitioned cages opposite infected conspecifics were able to detect the effects of infection in those individuals. This information caused physiological changes (elevated levels of serum corticosterone and increased kidney size) in these indirectly challenged dams. Treatment also led to changes in the adult behaviour of sons, with those that had developed in mothers exposed to ambient cues indicating threat of disease showing lower levels of aggression as adults. Most importantly, these sons then showed a different response to infection with *B. microti*, peaking and clearing infection earlier than individuals that had developed in mothers that did not have diseased neighbours.

Corticosterone is the primary glucocorticoid found in rats and mice, and is homologous to cortisol in humans and many other mammals (Edwards & Burnham 2001). Elevated levels of glucocorticoid reflect physiological changes associated with stressful stimuli (De Kloet *et al.* 2005), including social information about distress (Boissy *et al.* 1998) and immune responses (Fernandes 2000) in conspecifics. Therefore, the patterns seen here are consistent with the conclusion that the females had detected disease in neighbours. This detection did not appear to illicit an immune response in the *Babesia*-treatment dams (there was no effect on IgG production or spleen growth). The unusual kidney growth seen is difficult to explain, but may be the result of the kidney's proximity to the adrenal gland, which was overacting to produce high levels of corticosterone in these females. We did not see any corresponding behavioural indicators of stress in the behaviour of the dams during the stimulus phase (see the electronic supplementary material) and no aversive or avoidance behaviours were observed (O. Curno 2006, personal observation).

As the females in this study were exposed to auditory, behavioural, visual and olfactory information about their neighbours, a number of cues may have been used to detect disease in the stimulus males. There is no known effect of infectious disease on mouse vocalizations, but with increasing understanding of the complexity of mouse 'song' (Holy & Guo 2005) such a relationship may emerge, and may have provided an indicator for dams in this experiment. Behavioural cues may also have played a role, but *B. microti* at the inoculation levels we used has few recorded effects on host behaviour (Barnard *et al.* 1996), and we found no differences in the behaviour between infected and uninfected stimulus males (see the electronic supplementary material). The eyes and ears of laboratory mice become marginally paler at peak infection due to mild anaemia (Homer *et al.* 2000), but it is unlikely that this would have been an important cue given the poor eyesight of albino mice. Disease status is perhaps most likely to have been assessed via olfactory cues (Gelperin 2008).

In experiments in which only urine was offered to subjects, mice were able to detect infection in conspecifics (Penn & Potts 1998; Penn *et al.* 1998; Kavaliers *et al.* 2003). The underlying mechanism is currently unknown, but there are various hypotheses. Infections can change the composition of commensal microbes that play an important role in individual odour (Penn & Potts 1998; Hurst *et al.* 2001). Infection also leads to increased expression of MHC molecules (influencing the concentration of volatile acids in the urine) and changes the concentration of excreted endocrine by-products (Penn & Potts 1998). Antigens, antibodies and elevated levels of NO have been found in the urine of malaria patients (a similar intraerythrocytic parasite to *Babesia*) (Rodriguez-del Valle *et al.* 1991; Anstey *et al.* 1996). In our study, females may have also detected components of the parasites shed in the faeces, or subtle changes in the stimulus males' behaviour that we were not able to detect. Thus, the females in this study had access to a broad range of social information with which they could determine the disease status of their neighbours, with important consequences for their own physiology and ultimately the development of their male offspring.

Male offspring that developed in the *Babesia*-treatment environment were less aggressive as adults. Pre- and post-natal maternal corticosterone levels have been shown to affect the behaviour of offspring as adults via *in utero* exposure through the blood or post partum through milk (Edwards & Burnham 2001). Improved learning, reduced anxiety, sleep disturbances, enhanced fear, reduced social interactions and decreased exploratory behaviour have all been found as a result of endogenously or exogenously elevated corticosterone (or cortisol) (Edwards & Burnham 2001) during development. In humans, maternal stress during pregnancy is associated with a range of physical and behavioural effects in children and adult offspring (Edwards & Burnham 2001; Gluckman *et al.* 2005; Rickard & Lummaa 2007). Maternal stress during pregnancy may affect offspring development through an effect on foetal hormone production. Maternal stress has been shown to reduce the normal testosterone surge during development in male rat foetuses with downstream effects on adult male sexual behaviour (Pollard & Dyer 1985; Ward *et al.* 2003). It is therefore possible that the behavioural changes found here among the adult offspring of *Babesia*-treatment mothers were mediated by maternal corticosterone through foetal testosterone modulation. Interestingly, other studies have also found that offspring behavioural changes due to maternal stress are not evident before weaning in either the mother or offspring, but emerge later when the offspring reaches adulthood (Casolini *et al.* 1997), as was the case in our study (see the electronic supplementary material).

Table 1. Principal component analysis of infection profile, showing loadings derived from analysis of the time course of the infection. Scores for different treatments are shown in figure 3*b*.

	component 1
eigenvalue	2.652
percentage of variance	53.032
loadings	
day of onset of infection[a]	0.943
day of peak of infection	0.921
duration of infection (onset to clearance)[b]	0.724
time to clearance (from inoculation)	−0.454
infection level at the first clearance in the population	0.428

[a]Onset measured as the day on which infected red blood cells were first seen in blood smears.
[b]Clearance measured as the first day on which no red blood cells were infected following an infection peak.

Aggression in adult mice is associated with social dominance, territory acquisition and maintenance, and consequently increased access to mating opportunities (Barnard *et al.* 1994; Meagher *et al.* 2000; Waterman 2007). However, there is evidence that the benefits of aggressive behaviour are counterbalanced by costs associated with reduced resistance to disease (Barnard *et al.* 1994; Whitacre 2001; Zala *et al.* 2008*b*). In our study, male offspring that developed in a *Babesia*-treatment environment showed both accelerated response to infection (particularly an accelerated resolution phase; figure 3), and reduced aggression in novel social groups. Our results thus support the existence of a trade-off between social dominance and disease resistance. Evidence from other studies strongly implicates testosterone in the mediation of dominance–resistance trade-offs (Folstad & Karter 1992; Barnard *et al.* 1994; Whitacre 2001; Decristophoris *et al.* 2007). Of particular relevance in the context of our study, testosterone is known to interact with CD4+T-cells (Roberts *et al.* 2001), which are important for the resolution phase of *B. microti* infection (Homer *et al.* 2000). However, as we were not able to detect a significant effect of treatment on testosterone levels (see the electronic supplementary material), we cannot confirm a direct role for this hormone in the trade-off that we appear to have observed. Nevertheless, our results do point to a role for maternal corticosterone in the response to exposure to diseased conspecifics. Elevated levels of corticosterone may have led to the observed decrease in costly aggressive behaviour and the altered immune response seen in the male *Babesia*-treatment offspring. These results are consistent with evidence from the

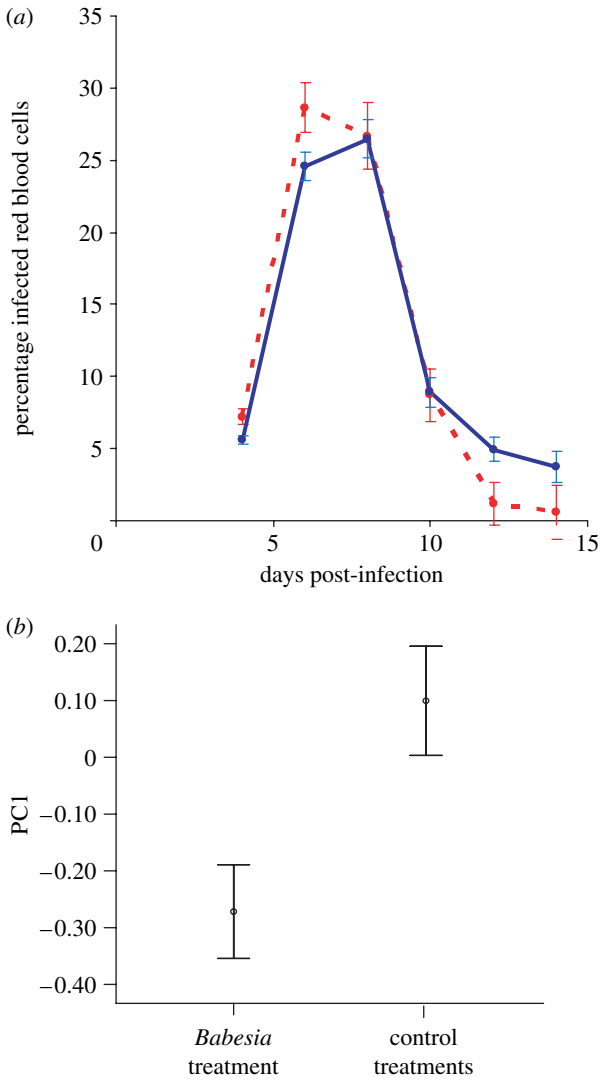

Figure 3. Treatment effects on adult offspring infection profiles. (*a*) The time course of infection with *B. microti* in adult offspring of dams exposed during pregnancy to infected (red dashed line) or control (blue solid line) males. (*b*) Scores for the first component (PC1) extracted by principal components analysis of five characteristics of the infection profile for offspring from (*a*) *Babesia*- and (*b*) control-treatment dams (see table 1: high positive scores reflect later onset, peak and clearance of infection; $n=145$ male offspring from 37 dams). Error bars represent ± 1 (s.e.).

literature on birds, where maternal hormones are increasingly recognized to be one of the most important factors mediating transgenerational immune priming (Tschirren *et al.* 2004; Groothuis *et al.* 2005*b*; Müller *et al.* 2005).

We suggest that our study demonstrates adaptive investment in immunocompetence (Manz *et al.* 2005; clearing infection sooner) in a situation where the imminent threat of infection has been perceived, possibly at the expense of investment in the acquisition of dominance. Our experimental design limits our ability to speculate on the specificity of the response we have observed, and it would be interesting to investigate in further experiments the extent to which exposure of mothers to infected conspecifics generates a more generalized altered sensitivity to all stressors or infections in their offspring. Whether general or specific, accelerated clearance of infection would return individuals to a competitive (Kilpimaa *et al.* 2004) and attractive (Hamilton & Zuk 1982; Ehman & Scott 2002) state more quickly, and thus enable them to secure future mating opportunities. Such benefits could outweigh any costs associated with reduced social dominance in an environment, where the risk of disease is high. Alternatively, the responses seen may not have been a response by offspring to a threat of disease specifically, but a generalized adaptation of life-history strategy in the presence of maternal stress. Either way, we conclude that either dams (through strategic maternal investment; Marshall & Uller 2007) or offspring (through individual life-history 'decisions') responded adaptively to an ambient threat of infection in order to maximize the chances of offspring survival (Hazel *et al.* 2000), and ultimately reproductive success.

To our knowledge, this study provides the first evidence for transgenerational regulation of immune response based on social information, and the implications of our findings are wide-reaching. These results are of great significance for our understanding of the role of parasites in the evolution of life histories (Virgin 2007), adding maternal perception of disease risk in the immediate environment to the factors potentially determining future social dominance, and related aspects of fitness, in offspring. Furthermore, the individual differences in disease susceptibility found within many species, including humans (Bateson *et al.* 2004; Rickard & Lummaa 2007), which are known to detect disease from the odours of others (Penn & Potts 1998), might be explained in part by similar maternal effects (Zinkernagel 2000). Through immunological maternal effects, individual decisions may have population-level consequences in the following generations (Mitchell & Read 2005), adding a new complexity to our understanding of epidemiological processes.

Moreover, our findings have implications for both animal welfare and the validity of scientific procedures. Most animal housing units enable some level of auditory and olfactory interaction between individuals. Here, we have shown that a measurable perception of disease in neighbouring conspecifics occurs, and elicits a stress response in the perceiver. Therefore, our results highlight the potential for substantial and unexpected effects of experimental design on animal welfare. Given this, we support the call for animal welfare sections in our publications (Würbel 2007) with the added suggestion that the welfare of bystanders, as well as experimental subjects, should be considered when planning experimental work. Finally, we begin to question the accuracy of considering co-housed 'control' animals as 'untreated' in scientific procedures, when in fact we have shown that they may respond in complex physiological and behavioural ways to ambient information from their treated neighbours. Further work to establish the mechanistic basis for our results, and the extent to which they can be generalized, is thus very desirable.

All the procedures were conducted under UK Home Office License and with approval from the appropriate government and university animal ethics committees (Animals (Scientific Procedures) Act: code of practice for the housing and care of animals used in scientific procedures, 1989).

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