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. 2008b). 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. 2008a). 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. 2005a,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.
(Barnard et al. 2005; Barnard & Behnke 2006). Babesia species are tick-borne haemosporidian 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 parasitaemia, which are quickly cleared (clearance by interferon (IFN-γ)) and then rapidly declining due to the action of CD4+ T cells and IFN-γ (Homer et al. 2000). Antibodies can block (Igarashi et al. 1999). 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, tumour necrosis factor-alpha (TNF-α), nitrous oxide (NO) and reactive oxygen species (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 (48×15×13 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; 28×45×13 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 (infestation 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. 1997a, 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 (χ²-test: χ² = 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 allo-grooms, 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: F₁,143 = 0.612, p = 0.691; PC1 score GLM: F₁,143 = 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 5×10⁷ 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).

(b) 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.
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 2c, 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: $F_{1,33} = 1.708, p = 0.184$). There was no effect of treatment on offspring IgG (repeated-measures GLM: $F_{1,33} = 1.021, p = 0.397$).

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 3a; repeated-measures GLM: $F_{3,30} = 2.612, p = 0.045$). 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 3b, 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 proximate to the adrenal gland, which was overacting to 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).

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 2a, 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 2b, 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: $F_{1,34} = 1.537, p = 0.224$).
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 intracellular 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; Ghuckman 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).

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. 2008b). 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 disease 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

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 3b.

<table>
<thead>
<tr>
<th>component 1</th>
<th>eigenvalue</th>
<th>percentage of variance</th>
</tr>
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<tbody>
<tr>
<td>loadings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day of onset of infection a</td>
<td>0.943</td>
<td>53.032</td>
</tr>
<tr>
<td>day of peak of infection</td>
<td>0.921</td>
<td></td>
</tr>
<tr>
<td>duration of infection (onset to clearance) b</td>
<td>0.724</td>
<td></td>
</tr>
<tr>
<td>time to clearance (from inoculation)</td>
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<td></td>
</tr>
<tr>
<td>infection level at the first clearance in the population</td>
<td>0.428</td>
<td></td>
</tr>
</tbody>
</table>

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.
generalized altered sensitivity to all stressors or infections. Mothers to infected conspecifics generates a more profound stress and elicits an immediate threat of infection has been perceived, possibly increasing the 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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REFERENCES


Kavaliers, M., Fudge, M. A., Colwell, D. D. & Choleris, E. 2003 Aversive and avoidance responses of female mice to


Chicago, IL: Chicago University Press.


Würbel, H. 2007 Publications should include an animal-welfare section. Nature 446, 257. (doi:10.1038/446257a)

