Effects of fatherhood on immune cell count and viability in the California mouse

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ABSTRACT

Females of several species show decreased ability to mount an immune response while rearing offspring; however, virtually no attention has been given to the trade-off between parenting and immunity in their male conspecifics. We tested the hypothesis that fatherhood may reduce a male’s ability to properly maintain the immune system in a biparental mammal. Male California mice (Peromyscus californicus) were placed into three different breeding conditions (n=8 per group): breeding males produced and raised offspring with a female; non-breeding males were paired with a tubally ligated female; and virgin males were housed in male pairs with no direct female contact. Eleven to fourteen days after the birth of a breeding pair’s first litter of pups, age-matched males from each condition were euthanized, and their spleens and thymuses were collected and mashed through 40 μm cell strainers. Splenic and thymic cells were counted on a hemocytometer and stained with carboxylfluorescein diacetate succinimidyl ester (CFSE) and fixable viability dye and subsequently quantified and analyzed using flow cytometry to assess cell viability (i.e. the proportion of live and dead cells). Breeding males had significantly higher splenic cell counts than virgins even when controlling for the body mass of the animals (p<0.05), though no significant differences in splenic cell viability were found. Across breeding conditions, no significant differences were found in thymic cell count or viability. These results provide indirect evidence that fatherhood may improve immune system function.

Keywords: Immune maintenance; trade-off; life-history theory; paternal investment; Peromyscus

FACULTY MENTORS

Wendy Saltzman
Department of Sociology

Professor Saltzman’s research focuses broadly on the integrative biology of stress and reproduction, including interactions between the two. Her research lab investigates proximate mechanisms underlying the expression of parental care, as well as the effects of parenthood on parents. They are especially interested in the causes and consequences of paternal care by father, and they focus on neural, hormonal, and sensory mechanisms that contribute to the activation of paternal behavior, as well as possible effects of fatherhood on stress responsiveness, anxiety, energetics, metabolism, and immune function in fathers.

Emma Wilson
Department of Biomedical Sciences

Professor Wilson’s primary focus is the immune response in the brain following Toxoplasma gondii infection. This is a common parasitic infection of many mammals including humans where prevalence is 10-30% in the USA and up to 80% in parts of Europe and South America. Infection with this parasite leads to an acute systemic inflammatory response that is controlled resulting in a chronic phase of infection where the parasite is maintained predominantly as a slow replicating form in the central nervous system.
INTRODUCTION

Life-history theory posits that allocating resources to some organismal process (e.g., growth, bodily maintenance) necessarily limits resources available for other processes. Reproduction, including producing and caring for offspring, can be particularly costly. While much research has focused on the trade-offs associated with maternal care of offspring, paternal behavior can also incur significant costs in the 5-10% of mammalian species known to be biparental. Male prairie voles (Microtus ochrogaster), for instance, experience lower survivorship when living in male-female pairs than when living singly. Biparental rodents and primates also endure decreases in body mass and fat reserves in association with fatherhood.

Studies in the bird the great tit (Parus major) suggest that the energy demands inherent in paternal care can make fathers susceptible to parasitism, presumably due to dampened immune function. Experimentally increasing clutch sizes resulted in a higher number of haematozoan parasites in males. Further, offspring-feeding effort and malarial infection were higher in males raising enlarged broods; brood size did not affect feeding behavior or infection frequency in females.

Hormones associated with both reproduction and immune function might mediate potential trade-offs between these processes. Estrogen and testosterone implants promote paternal behavior in the biparental California mouse (Peromyscus californicus), and estrogen has been shown to prevent apoptosis in T helper cells, while testosterone promotes T cell death through binding to receptors on the thymic epithelium. It has also been posited that leptin, a hormone secreted by adipose tissue that affects T cell and macrophage proliferation, may play a role in mediating the trade-off between reproduction and immunity. The surgical removal of fat tissue decreases immune capability in hamsters and voles, and circulating leptin concentrations are significantly lower in prairie vole fathers compared to non-fathers.

The interactions between reproduction and immunocompetence are nuanced and, in the case of males particularly, poorly understood. To our knowledge, no study has addressed the effects of fatherhood on immune function in mammals. Therefore, we tested the hypothesis that fatherhood reduces males’ ability to maintain the immune system. We predicted that fathers would have fewer lymphocytes and lower proportions of live to dead lymphocytes in two immune organs, the spleen and thymus, compared to non-fathers.

MATERIALS AND METHODS

Animals

California mice born in our colony (University of California, Riverside, CA) and descended from animals purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC) were used in this study. Peromyscus californicus provides a valuable model species, as it is biparental and monogamous. All animals were housed in 44 x 24 x 20 cm polycarbonate cages with aspen shavings as bedding and cotton as nesting material. Prior to the start of the experiment, mice were raised in their parents’ cage until 27-35 days of age, upon which they were ear-punched and weaned into cages of 3-4 same-sex, age-matched animals. The colony was maintained at ambient temperature of approximately 18-26°C, humidity of approximately 60-70%, and a 10:14-hour light schedule (lights on at 0500h and off at 1900h). Food (Purina Rodent Chow 5001) and water were freely available. All procedures were approved by the University of California, Riverside Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Breeding Condition

At 195-225 days of age, sexually mature male California mice were randomly assigned to one of three breeding conditions: breeding males (N=9) were pair-housed with an unrelated female that subsequently produced offspring; non-breeding males (N=8) were pair-housed with a female rendered unable to reproduce by tubal ligation as a control for any extraneous effects of cohabitation; and virgin males (N=10) were pair-housed with an unrelated male from their original, post-wean cage.
Tubal ligations were performed under isoflurane anesthesia in sterile conditions 10 days prior to pair formation. All animals were weighed twice weekly to assess changes in body mass, monitor pregnancy in females, and habituate animals to handling.

**Organ Preparation**

Eleven to fourteen days after a breeding pair gave birth, age-matched males from each condition were euthanized by decapitation; the spleens and thymuses were extracted and placed in separate 50 ml conical tubes of sterile RPMI complete media (Sigma-Aldrich, St. Louis, MO). Organs were passed through 40 micron cell strainers (BD Falcon, San Jose, CA) to create single-cell suspensions for later staining. Cells were treated with 0.86% lysis buffer (Invitrogen, Eugene, OR) on ice for 5 minutes, washed in cRPMI media, counted on a hemocytometer to assess total cell counts, and resuspended in fluorescence-activated cell sorting (FACS) buffer to a concentration of 2 million cells/ml.

**Carboxylfluorescein Diacetate Succinimidyl Ester and Fixable Viability Dye Staining**

Carboxylfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Eugene, OR) and eFluor Fixable Viability Dye (eBioscience, San Diego, CA) were used to determine the proportion of live and dead cells, respectively. Splenic and thymic cells were incubated with CFSE (2 μL/ml cells) for 10 minutes and quenched with chilled cRPMI. After washing with phosphate-buffered saline (PBS), cells were incubated with viability dye (1 μL/ml cells) for 30 minutes. Unlabeled cells were used to control for auto-fluorescence, and cells stained with either CFSE or viability dye served as compensation controls. Positive controls for viability dye were created by killing cells with paraformaldehyde (PFA), after which the membranes were repermeabilized using 0.3% saponin in FACS buffer. All samples were fixed with 4% PFA in PBS for later analysis with flow cytometry.

**Analysis**

Fluorescently labeled samples were analyzed with flow cytometry (FACSCanto II; BD, San Jose, CA). Cytometry data were processed and analyzed using DiVA (3ivx Technologies, Sydney, Australia) and FlowJo v.8.7.3 (TreeStar, Ashland, OR). Carboxylate microspheres (10 μL / 1 million cells; Polysciences, Warrington, PA) were added to each sample to allow for calibrated event counts prior to analysis with flow cytometry. Body masses were analyzed by ANOVA, and proportions of viable cells were analyzed by ANOVA using SPSS (v.20; IBM, Somers, NY). Cell counts were normalized by log transformation and analyzed by ANCOVA using Stata 1C (v.10; StataCorp, College Station, TX). Appropriate post hoc tests were applied where necessary.

**RESULTS**

**Splenic and Thymic Cell Counts**

Splenic cell counts were significantly affected by breeding condition (Tables 1, 2). Tukey’s Honestly Significant Difference post hoc test indicated that breeding males had significantly higher counts than virgin males (p<0.05), but not non-breeding males (Fig. 1). This effect was not solely attributable to body mass, as body mass at the time of sacrifice did not differ among housing conditions. Thymic cell counts did not vary significantly across conditions (Tables 1, 2).

![Fig. 1. Breeding males had significantly higher splenic cell counts than virgin males (* p<0.05).](image-url)
The proportion of viable to non-viable cells did not differ significantly across breeding conditions in either spleen or thymus (Table 1).

We originally predicted breeding males to have lower immune cell counts and proportionately lower live cell counts compared to non-breeding and virgin males. Instead, we found that breeding males had significantly more splenic cells than did virgin males, but no differences in thymic cell counts or in proportions of viable cells in either organ. A previous study in our lab showed that fathers had heavier thymuses than both non-breeding males housed with a tubally ligated female and virgin males housed with another male. Together, the findings of these two studies suggest that fatherhood may promote cell proliferation or prevent cell death in the organs of the immune system. The thymus is the site of maturation for T cells, a population of lymphocytes that recognize and destroy foreign antigens through direct contact and the release of cytotoxic factors. The spleen, while not immunologically active itself, filters lymph, which carries immune cells; increases in cell counts in the spleen are suggestive of increases in the total number of immune cells in an organism, though not definitively so.

The cost of paternal care under controlled laboratory conditions may not be biologically expensive enough to require trade-offs with immune-system maintenance. Klasing (2004) estimates that the cost of maintaining the immune system in terms of lysine, an amino acid indicator of metabolic demand on an organism, amounts to only 3% of the total bodily requirement in chickens. However, increases in the number of immune cells may be evolutionary advantageous. Pup survivorship is increased by elevated foraging behavior in California mouse fathers; therefore, enhanced immune function during periods of infant care may assist fathers in defending against increased exposure to foreign antigens during foraging. It is possible that a more pronounced difference between fathers and non-reproductive males might be seen if males are subjected to an antigenic challenge; the production of acute-phase proteins during inflammation appears to be the most expensive metabolic process besides somatic growth. In our own laboratory, California mouse fathers undergoing antigenic challenge via injection with lipopolysaccharide (derived from the outer membrane of gram-negative bacteria) display more pronounced sickness behavior and larger changes in body temperature as compared to identically treated virgin males, suggesting that fathers have a more robust immune response. However, non-breeding males in the present study had a non-significant, intermediate splenic cell count.

**Cell Viability**

The proportion of viable to non-viable cells did not differ significantly across breeding conditions in either spleen or thymus (Table 1).

**DISCUSSION**

We originally predicted breeding males to have lower immune cell counts and proportionately lower live cell counts compared to non-breeding and virgin males. Instead, we found that breeding males had significantly more splenic cells than did virgin males, but no differences in thymic cell counts or in proportions of viable cells in either organ. A previous study in our lab showed that fathers had heavier thymuses than both non-breeding males housed with a tubally ligated female and virgin males housed with another male. Together, the findings of these two studies suggest that fatherhood may promote cell proliferation or prevent cell death in the organs of the immune system. The thymus is the site of maturation for T cells, a population of lymphocytes that recognize and destroy foreign antigens through direct contact and the release of cytotoxic factors. The spleen, while not immunologically active itself, filters lymph, which carries immune cells; increases in cell counts in the spleen are suggestive of increases in the total number of immune cells in an organism, though not definitively so.

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**Table 1.** Mean ± SE splenic and thymic cell counts in millions (standardized for mean body mass of 48.44 g) and mean ± SE proportion of viable splenic and thymic cells in breeding, non-breeding, and virgin males. Statistically significant p values (<0.05) are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Breeding males</th>
<th>Non-breeding males</th>
<th>Virgin males</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenic cell count</td>
<td>17.23 ± 1.73</td>
<td>14.15 ± 1.83</td>
<td>11.82 ± 1.63</td>
<td>0.029</td>
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<tr>
<td>Splenic cell viability</td>
<td>0.9334 ± 0.0328</td>
<td>0.9205 ± 0.0417</td>
<td>0.9166 ± 0.0309</td>
<td>0.095</td>
</tr>
<tr>
<td>Thymic cell count</td>
<td>19.71 ± 6.24</td>
<td>32.74 ± 5.81</td>
<td>24.09 ± 5.16</td>
<td>0.259</td>
</tr>
<tr>
<td>Thymic cell viability</td>
<td>0.9256 ± 0.0384</td>
<td>0.9245 ± 0.0354</td>
<td>0.9312 ± 0.0319</td>
<td>0.904</td>
</tr>
</tbody>
</table>

**Table 2.** ANCOVA results of splenic and thymic cell counts controlling for body mass. Housing condition significantly affected splenic cell counts. Statistically significant p values (<0.05) are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Factor</th>
<th>df</th>
<th>F</th>
<th>p</th>
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</thead>
<tbody>
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<td>Body mass</td>
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<td>16.08</td>
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<tr>
<td>Thymic cell count</td>
<td>Housing condition</td>
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<td>0.52</td>
<td>0.600</td>
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<tr>
<td></td>
<td>Body mass</td>
<td>1</td>
<td>6.27</td>
<td>0.021</td>
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</table>
between breeding and virgin males, suggesting that pair bonding or cohabitation with a female alone may also have some effect. Additional research is required before firm conclusions can be drawn about the social and reproductive influences on immune function in male California mice.

Aside from metabolic cost, the hormonal changes that accompany paternal status may not be pronounced enough to induce significant reductions in the immune system. Pregnancy in BALB/c mice has been shown to reduce the number of both newly formed and committed B lymphocytes, an effect mimicked in non-pregnant mice by estrogen treatment. Natural hormonal changes in males, conversely, may prevent immune cell death, allowing for a more effective immune response. Further studies are required to elucidate the mechanism of this effect and to determine if similar enhancement of the immune system is seen in fathers in other biparental species.

REFERENCES


