Birds rearing experimentally enlarged broods have lower antibody responses to a novel antigen, and we tested three hypotheses that could explain this result. We used zebra finches *Taeniopygia guttata* inoculated with sheep red blood cells (SRBC) as a study system, for which this trade-off was previously demonstrated. 1. Compensatory cellular immunity: The humoral immune response is slow, and removal of SRBC through up-regulated cellular immunity could pre-empt an antibody response. However, cellular immune response to PHA decreased with increasing brood size, allowing rejection of this hypothesis. 2. Costs of antibody-production: Chicks in large broods grow less well, and birds with large broods may allocate resources to chicks instead of antibodies when these are costly. Compared to saline controls, SRBC suppressed metabolic rate in the hours following immunisation, but there was no effect in the following night, or at any time 4 and 8 days later. Fitness costs were measured by repeatedly immunising parents with SRBC while rearing young. Chick growth, parental condition, and subsequent reproduction of the parents were not affected by SRBC. We conclude that the costs of antibody formation cannot explain the trade-off between brood size and antibody responsiveness. 3. Costs of immune system maintenance: Maintaining a system enabling antibody-formation may be very costly, and birds rearing large broods may have down-regulated this system. Based on this hypothesis we predicted that antibody formation would still be reduced in parents rearing large broods when immunised after rearing the chicks. Our results confirmed this prediction, and we suggest that birds rearing large broods have lower antibody responses because they economised on the maintenance costs of the immune system.
Understanding why parents rearing experimentally enlarged broods have lower antibody (ab)-responses may yield more general insights in factors determining resistance to parasites, and thereby be relevant for understanding host-parasite interactions. We used zebra finches *Taeniopygia guttata* ( Vieillot, 1817) inoculated with sheep red blood cells (SRBC), in which a trade-off between brood size and antibody-responsiveness has previously been demonstrated (Deerenberg et al. 1997), and tested three hypotheses that could explain reduced ab-responses in birds rearing large broods:

1. Compensatory cellular immunity: The humoral immune system is slow, taking several days to produce measurable levels of antibodies. In the mean time antigens are also attacked and removed by the cellular immune system (Roitt et al. 1996). There may be a phenotypic trade-off between different components of the immune system, and when down regulation of humoral immunity is accompanied by up-regulation of cellular immunity (Gross et al. 1980, Gustafsson et al. 1994, Cotter et al. 2004), the latter could theoretically result in removal of antigens before antibodies are produced. Thus enhanced cellular immunity could explain lower ab-production of birds rearing large broods. We compared cellular immunity (PHA-responsiveness) between birds rearing small and large broods to test this hypothesis (Experiment 1).

2. Costs of ab-production: Resource competition between offspring and self-maintenance could lie at the heart of the trade-off between brood size and ab-responsiveness when ab-production entails a significant cost. We investigated the costs of ab-production in two experiments. In experiment 2A we quantified the consequences of inoculation with SRBC for energy expenditure (Demas et al. 1997, Svensson et al. 1998). From an evolutionary perspective only fitness costs are relevant. We therefore examined the effect of repeated SRBC inoculations of breeding birds on offspring growth and antibody-responsiveness. There may be fitness costs. We investigated the costs of ab-production in two experiments. In experiment 2B we quantified the consequences of inoculation with SRBC for energy expenditure (Demas et al. 1997, Svensson et al. 1998). From an evolutionary perspective only fitness costs are relevant. We therefore examined the effect of repeated SRBC inoculations of breeding birds on offspring growth and antibody-responsiveness. There may be fitness costs.

3. Costs of immune system maintenance: Costs of an ab-response could be low, but maintaining an immune system required to produce high ab-responses may be costly (Fair et al. 1999, Williams et al. 1999, Schmid-Hempel 2003), and birds rearing large broods may have down regulated the immune system to a lower activity level. On the basis of this hypothesis we predict that ab-formation would be reduced in parents rearing large broods when immunised after the chicks had been raised (and there is no longer a trade-off between resources allocated to offspring and self-maintenance) because recovery of the immune system requires time. We tested this prediction (Experiment 3).

### Methods

#### Housing and breeding conditions

Birds were housed in 80 × 40 × 40 cm (l × h × d) cages, with two perches, water, cuttlebone, and food ad libitum (mixture of tropical and grass seeds). A protein supplement was provided three times per week, except during the experiments (in experiments with breeding birds protein supplementation was stopped when egg laying started). Rooms were approximately 22 °C, humidity was > 60%, and lights were on 14 h per day (6:30 to 20:30). Breeding pairs were supplied with a nest box and nest material. Nests were checked regularly to monitor the start of laying, clutch size and the number of hatchlings. Chicks were transferred between broods 1–3 d after hatching to create broods with 2 or 6 young (experiment 1), or 2, 4 or 6 young (experiments 2B and 3). In experiment 2B, but not in experiments 1 and 3, there was still a correlation between original and manipulated brood size, due to the low numbers of broods available simultaneously. As in earlier brood size manipulation studies (Deerenberg et al. 1996), there were significant effects of brood size on chick growth in all experiments, but for brevity we do not report these data.

#### Cellular and humoral immunity

A humoral immune response was induced with an intraperitoneal injection of 0.1 ml 2% SRBC suspended in phosphate buffered saline (PBS) as described by Deerenberg et al. (1997). Controls were injected with PBS only. Blood samples (100–150 µl) were taken from the *Vena jugularis*, centrifuged at 12 477 g for 10 min, and plasma was stored at −20 °C until analysed. Antibodies were measured in duplicate using haemagglutination (Hudson and Hay 1989). Titers were scored as the inverse of the highest dilution at which antibodies were detectable and are based on a base 2 log scale. Blood samples were taken six days after immunisation, when antibody titers reach maximum values (our unpubl. obs.). Birds injected with PBS had no measurable SRBC antibodies (Deerenberg et al. 1997; our unpubl. obs.). Only primary immune responses are reported in this paper.

A cellular immune response was induced by injecting 40 µg phytohaemagglutinin (PHA-P, L8754, Sigma, in 0.2 ml PBS) intradermally into one wing web. PHA is
mitogenic to T-lymphocytes. The other wing web was injected with 0.2 ml PBS. Before injection and after 24 h we measured the thickness of both wing webs to the nearest 0.01 mm using a spessimeter (Mitutoyo, 2046F-60). The PHA response was calculated by subtracting the change in thickness of the PBS injected wing from the change in thickness of the PHA injected wing (Smits et al. 1999).

**Metabolic rate**

During measurements birds were in a transparent Plexiglas chamber of 21.6 l. Food was present *ad libitum*. No water was provided to facilitate drying the air. Light regime and temperature were similar to standard housing conditions. Birds were weighed and moved in or out of the respirometer between 12:00 h and 15:00 h. We used measurements obtained between 16:00 h and 12:00 h the following day.

Air flow through the respirometer chambers was set to ca. 18 l/h in experiment 2 when there was one bird per chamber, and to ca. 24 l/h when there were two birds per chamber. Air flow was measured and controlled with mass-flow controllers (Brooks Instrument, Model 5850E, accuracy 0.02%). Air was dried with molecular sieve (8Å, Merck) before oxygen measurement with an AMETEK Applied Electrochemistry analyser (model S-3A/II, accuracy 2%) and carbon dioxide measurement with an infrared analyser (BINOS-IR 1.2, accuracy 2.5%). Accuracy values according to manufacturer's specifications. Oxygen consumption was calculated according to Hill (1972), and metabolic rate was calculated using the RQ dependent energy equivalent of the oxygen consumed (kJ/L O₂) following Schmidt-Nielsen (1997).

Passive infrared (PIR) sensors recorded movement 340 times per 360 seconds. We defined 'activity' as the proportion of samples with movement. Individual sensors differed in sensitivity, and in the second session in experiment 2A we corrected for these differences prior to analysis. In the first session of experiment 2A birds were always measured with the same PIR sensors and because we made within individual comparisons no correction was necessary.

**Statistical analyses**

Data were analysed with standard techniques (t-tests, Mann Whitney U-test, general linear models with normal or binomial error distribution) using the computer package Statistix. Responses of pair-members were not correlated in any of the tests when experimental treatment was controlled for statistically, and pair members were therefore treated as independent samples.

**Experiment 1: Compensatory cellular immunity**

Broods with two (n=10) or six (n=9) nestlings were created. PHA/PBS was injected in both parents when the chicks were 17 d old. One female died due to unknown causes before the PHA response was measured, and one measurement failed, presumably because the injection was not successful (Fig. 1). This case was removed from the analysis, but this did not change the result.

**Experiment 2: Costs of ab-response**

**Experiment 2A: energy costs**

The energy costs of an antibody response was measured in two separate immunisation sessions, which differed mainly in the timing of metabolic measurements relative to immunisation. In the first session, 20 males were housed individually, of which 10 were injected with SRBC and 10 with PBS. Metabolic measurements were performed 5 days before and for 20 h immediately following immunisation. In the second session birds were housed in single-sex pairs (n=16 pairs, 8 of each sex, 4 controls and 4 immunised). Metabolic measurements were done on the paired birds together (separated by wire mesh) to increase accuracy. Metabolic rate and activity were measured four times: six and two days before immunisation, and four and eight days after immunisation. The first measurement served to accustom the birds to the protocol, and is not further
discussed. The second measurement served as within-pair control.

**Experiment 2B: fitness costs**

Pairs of broods with the same brood size were selected, and in each dyad one randomly chosen pair was immunised three times with SRBC, on day 7, 9 and 11 after hatching. The other pair served as control and was injected with PBS on the same days. Nestlings were weighed before the first injection (day 7), and before blood sampling (day 13). When chicks are 7–13 d growth is approximately linear (our unpubl. data). After the young fledged the nest box was removed. The young were measured (mass, tarsus, wing length) and removed when 35 d old. One week later a second blood sample was taken from the adults, and all birds were immunised once with SRBC. The initial purpose was to investigate the build-up of immunological memory, but the antibody titers at the second immunisation were too high to provide a meaningful test, and the memory data will not be presented. After the final blood sample was taken, the pairs were again supplied with a nest box, and reproduction was monitored as before.

**Experiment 3: Costs of immune system maintenance**

Trios of broods were formed, and chicks were distributed so that independent of the original brood size in each trio there were broods with 2, 4 or 6 chicks. Nestlings and parents were weighed at the time of manipulation. At 16 d after hatching, the young were weighed and measured, and removed from the cage. The parents were also weighed at this time. One day later the parents were weighed again, and immunised with SRBC, and a blood sample was taken six days later.

**Results**

**Experiment 1: Compensatory cellular immunity**

PHA response was significantly weaker in birds rearing large broods (Fig. 1; \( F_{1,34} = 12.0, P = 0.001 \)), also when the sexes were analysed separately (females: \( F_{1,16} = 6.11, P = 0.025 \); males: \( F_{1,16} = 5.95, P = 0.027 \)). There was no difference between the sexes (\( F_{1,32} = 0.9, P = 0.3 \)) and no brood size x sex interaction (\( F_{1,32} = 0.08, P = 0.8 \)).

**Experiment 2: Costs of ab-response**

**2A energy costs**

Of the immunised birds in the first session (short term effects) 70% produced SRBC-antibodies, and mean antibody-titer was 5.1 (SE = 1.4, n = 10, including non-responders). Mass, metabolic rate and activity did not differ between control and immunised birds during the control measurements (t-tests, all \( P > 0.3 \)). We calculated the difference between the post-immunisation and individual control values measured 5 days before immunisation. SRBC reduced metabolic rate in the afternoon following injection (\( t_{18} = 2.16, P < 0.05 \)), but this effect disappeared over the next two time intervals (Fig. 2a; night: \( t_{18} = 1.14, P = 0.18 \); morning: \( t_{18} = 0.64, P = 0.53 \)). Analysis on a finer time-scale revealed that the decrease in metabolic rate had disappeared after approximately nine hours (Fig. 2c). An immunisation effect on activity can obscure metabolic effects (Gentry et al. 1997). However, activity levels in the afternoon and the next morning were not affected by immunisation (Fig. 2a; \( t_{18} < 0.2, P = 0.9 \)). Controlling statistically for the change in activity did not change the results.

All immunised birds in the second session (effects 4 and 8 days after immunisation) produced SRBC-antibodies. Mean antibody-titer was 4.47 (SE = 0.62, n = 16). Metabolic rate and activity (afternoon or morning) during the control measurements did not differ between experimental groups (t-tests; all \( t_{14} < 0.8, P > 0.5 \)). The change in metabolic rate (afternoon, night, and morning) was independent of immunisation at 4 and 8 days after immunisation (Fig. 2b; t-tests: all \( t_{14} < 1.14, P > 0.27 \)). Activity level was significantly reduced in immunised birds during the afternoon on day 4 after immunisation (Fig. 2a; \( t_{18} = 3.12, P < 0.008 \), but not at any other time (all \( t_{14} < 1.1, P > 0.3 \)). Controlling for changes in activity when testing immunisation effects on metabolic rate did not change the results.

**2B Fitness costs**

We used 16 control broods, and 15 experimental broods. In total, 28/30 (93%, sexes combined) of the immunised birds responded with the formation of SRBC-antibodies. We verified whether there was an association between brood size and ab-response. Because we took two samples (6 and 35 d after immunisation), we used repeated measures ANCOVA. Ab-response declined significantly with broods size in females (Fig. 3a; \( F_{1,12} = 6.3, P < 0.03 \)), but not in males (Fig. 3b; \( F_{1,12} = 0.6, P = 0.4 \)). The brood size effect was significant when sexes were pooled (\( F_{1,26} = 4.6, P < 0.05 \), and there was no significant sex x brood size interaction (\( F_{1,24} = 0.7, P = 0.4 \)). Brood size before and after manipulation were correlated in this experiment, and to compare effects of natural and artificial brood size variation we replaced brood size with brood size at manipulation and the number of young added/removed (these variables summed is the manipulated brood size). Both parameters approached significance (brood size at manipulation: \( F_{1,25} = 3.73, P < 0.07 \), number of chicks added/...
removed; $F_{1,25} = 3.14, P < 0.09$, but, more importantly, the slopes of these effects were indistinguishable (difference $B/5\%$).

Nestling mass at time of the first immunisation did not differ between control and immunised pairs (immunisation: $F_{1,28} = 0.01, P = 0.9$; controlling for brood size: $b = -0.34$ (SE = 0.13) g/young, $F_{1,28} = 6.9, P < 0.02$). Controlling for brood size, growth in the experimental period (age 7–13 d) was not affected by the immunisations (Fig. 4a; $b = 0.04$ (SE = 0.09) g/young/d, $F_{1,28} = 0.2, P = 0.7$). Nestling mortality in the experimental period was too low to warrant statistical analysis (3/122 chicks died). Chick mass at 35 d was also independent of immunisation (Table 1; repeated measures ANOVA: $F_{1,29} = 0.7, P = 0.4$). Thus there was no evidence of any effect of parental immunisation on development of the chicks.

The costs of an immune response could be paid through subsequent reproduction (e.g. via an effect on parental state), but neither clutch size, nor the interval between the first egg of a new clutch and the time a nest box was offered were affected by immunisation (Fig. 4c; laying interval: Mann Whitney-U-test, $P > 0.9$; clutch size: $t_{24} = 0.64, P = 0.5$). Controlling for previous brood size did not change these results.

**Mass-changes of immunised birds**

In experiment 2A mass change from the immunisation to the blood sampling 6 days later was independent of immunisation (session 1: $t_{18} = 1.56, P = 0.14$; session 2: $t_{18} = 1.41, P = 0.17$), and effects were opposite in the two sessions (Table 1). Looking at other intervals after immunisations, or analysing sexes separately, does not change these results (Table 1). In experiment 2B (brood rearing birds) there was also no significant effect of immunisation (Table 1; repeated measures ANOVA: $F_{1,24} = 0.64, P = 0.4$). Thus there was no evidence of any effect of parental immunisation on development of the chicks.
Experiment 3: Costs of immune system maintenance

We pooled the data from experiment 3 with the data from the control group of experiment 2B, in which birds were also immunised after the young were removed (see methods). There was a negative association between brood size and the proportion of birds forming SRBC-antibodies (Fig. 5; logistic regression: $F_{1,94} = 5.1, P < 0.03$). The brood size effect on ab-formation did not differ between experiments 2B and 3 (experiment: $F_{1,93} = 0.1, P = 0.8$; experiment x brood size interaction: $F_{1,92} = 0.1, P = 0.7$). Neither experimental effect, nor the mean level differed between the sexes (sex: $F_{1,93} = 0.2, P = 0.7$; sex x brood size interaction: $F_{1,92} = 0.1, P = 0.8$).

For comparison, the data of Deerenberg et al. (1997) are also shown in Fig. 5. The brood size effect did not differ significantly between the two studies (study: $F_{1,136} = 0.6, P = 0.4$; brood size x study interaction: $F_{1,135} = 0.4, P = 0.5$), and was highly significant in this combined analysis ($F_{1,137} = 9.9, P < 0.002$).

The persistence of the brood size effect on ab-responsiveness may be explained by an effect of brood size on body mass (data experiment 3 only). We controlled for sex in subsequent analyses; the interaction between sex and brood size was in no case significant. Parents with large broods lost more mass during brood rearing than parents with small broods (Fig. 6; $b = -0.16$ (SE $= 0.05$) g/young, $F_{1,62} = 12.3, P < 0.001$; sex: $F_{1,62} = 0.2, P = 0.6$). Mass gain over the 24 h after the brood was removed increased with brood size ($b = 0.08$ (SE $= 0.03$) g/young, $F_{1,62} = 7.2, P < 0.01$; sex: $F_{1,62} = 6.8, P < 0.02$). Consequently, body mass was independent of brood size and the proportion of birds forming antibodies (Fig. 5; logistic regression: $F_{1,94} = 0.2, P = 0.1$; sex: $F_{1,92} = 0.5, P < 0.03$). Further mass change until blood sampling was also independent of brood size (Fig. 6; $F_{1,62} = 0.1, P = 0.7$; sex: $F_{1,62} = 28.7, P < 0.0001$). Thus the persistence of the brood size effect on ab-responsiveness was not due to effects on mass.

Table 1. Mass (g, SE in brackets) at immunisation and subsequent mass change relative to mass at immunisation (time = 0).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sex</th>
<th>N</th>
<th>Treatment</th>
<th>Time (d) after immunisation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2A-1</td>
<td>Males</td>
<td>10</td>
<td>Control</td>
<td>15.6 (0.54)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Immune</td>
<td>15.9 (0.69)</td>
<td>0.12 (0.20)</td>
</tr>
<tr>
<td>2A-2</td>
<td>Males</td>
<td>8</td>
<td>Control</td>
<td>14.4 (0.56)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Immune</td>
<td>14.8 (0.68)</td>
<td>0.33 (0.14)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>8</td>
<td>Control</td>
<td>15.4 (0.88)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Immune</td>
<td>15.1 (0.67)</td>
<td>0.34 (0.53)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>16</td>
<td>Control</td>
<td>14.9 (0.52)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Immune</td>
<td>14.9 (0.46)</td>
<td>0.33 (0.26)</td>
</tr>
<tr>
<td>2B</td>
<td>Males</td>
<td>16</td>
<td>Control</td>
<td>13.6 (0.35)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Immune</td>
<td>14.2 (0.44)</td>
<td>0.09 (0.18)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>16</td>
<td>Control</td>
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</tr>
<tr>
<td></td>
<td>15</td>
<td>Immune</td>
<td>14.2 (0.38)</td>
<td>0.23 (0.07)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>32</td>
<td>Control</td>
<td>14.5 (0.37)</td>
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<tr>
<td></td>
<td>30</td>
<td>Immune</td>
<td>14.2 (0.28)</td>
<td>0.16 (0.09)</td>
</tr>
</tbody>
</table>
Our study was based on the effect of brood size on ab-responsiveness reported by Deerenberg et al. (1997), and it is therefore important that we were able to replicate this result. Practically all birds formed antibodies in response to repeated immunisation, but the strength of the response decreased with increasing brood size (Fig. 3). This extends the results of Deerenberg et al., because their result was restricted to a brood size effect on the proportion of responders. Cellular immunity also decreased with increasing brood size (Fig. 1), a result previously found only in pied flycatchers Ficedula hypoleuca (Moreno et al. 1999). Our finding that both cellular and humoral immunity decrease with increasing brood size indicates a general trade-off between reproductive effort and immunocompetence, as opposed to an effect evident in only parts of the immune system.

We tested three hypotheses that could explain why birds with large broods have lower ab-responsiveness. Compensatory cellular immunity is an unlikely explanation, because PHA responsiveness decreased with brood size. The metabolic consequences of SRBC were small, and restricted to the first few h after inoculation (Fig. 2). Furthermore, the direction of the effect was opposite to what we had expected, since the birds decreased their metabolic rate. This is however in agreement with the decrease in food intake of sick animals (Murray and Murray 1979), and with the symptoms of mild septic shock (Romanovsky et al. 1997). Energy costs of immune function could reveal itself through an effect on energy reserves (Ots et al. 2001), but no such effects were found. The lack of strong effects of immunisation on metabolic rate is in agreement with the results obtained in other avian species (Henken and Brandsma 1982, Siegel et al. 1982, Svensson et al. 1998, Hörak et al. 2003, but see Ots et al. 2001), suggesting that the metabolic costs of mounting a humoral immune response are negligible. This is in agreement with the results of theoretical calculations for poultry in which the costs of immune function were found to be small compared with growth and egg production (Klasing 1998).

Although the metabolic consequences of an immune response were small, the costs could be higher when measured in another currency, e.g. protein or amino acid turnover (Klasing and Austic 1984, Parry Billings et al. 1992, Lochmiller and Deerenberg 2000). Ultimately only fitness costs are relevant, but repeated inoculation of parents rearing young had no effect on parameters strongly related to fitness in free-living birds (Fig. 4). To our knowledge this is the first demonstration that the trade-off between brood size and humoral immunity cannot be explained by the costs of ab-production. Fitness costs of resistance may be expressed in harsh environments only (Kraaijeveld and Godfray 1997, Moret and Schmid-Hempel 2000), but reduced immune responses with increasing brood size indicates parental performance was constrained. This suggests that zebra finches rearing large broods are in situation comparable with a harsh environment. Immunopathological and oxidative damage may constitute a cost of immune function (Råberg et al. 1998, Westneat and Birkhead 1998, von Schantz et al. 1999), but these processes were apparently not of sufficient magnitude to cause fitness costs. Thus, at least in the context of our study, such damage is unlikely to be important. In contrast to the...
The costs of immune function may lie in the maintenance of a system enabling ab-formation, rather than in immune system deployment, and the trade-off between brood size and antibody-formation may be attributable to down-regulation of the capacity to produce antibodies in birds rearing large broods. The negative association between brood size and immune function persisted after brood rearing (Fig. 5), in agreement with this hypothesis. To our knowledge, these are the first data indicating that economising on maintenance costs may explain the trade-off between brood size and humoral immunity. Results from two experiments that differed in the length of the brood-rearing period and in the interval between chick removal and inoculation were indistinguishable. This suggests that the brood size effect on parental state does not disappear very rapidly (i.e. in days), which is in agreement with the observation that costs of reproduction can reveal themselves months after parental care has ended (Gustafsson and Sutherland 1988, Daan et al. 1996, Verhulst 1998).

An alternative explanation for the persistence of the brood size effect on ab-formation is that down regulation of immunity functions to avoid autoimmune reactions to circulating cell ‘debris’ that results from the tissue damage associated with a high work load (Råberg et al. 1998). However, independent support for the significance of the costs of immune system maintenance comes from artificial selection experiments on immune function, which reported negative associations between immune function and fitness related parameters (Verhulst et al. 1999, Schmid-Hempel 2003). Since these animals were not allocating resources to an induced immune response when fitness-parameters were measured, it follows that the fitness consequences were due to investment in the capacity to respond. Future studies of resource allocation to immune defence should therefore distinguish between the maintenance and deployment costs of the immune system.

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