# Transgenerational effects of maternal immune challenge in tree swallows (*Tachycineta bicolor*)

**G.A. Lozano and R.C. Ydenberg**

**Abstract**: The fact that avian eggs contain antibody of maternal origin is well documented, but only recently has this phenomenon been considered in an ecological context. We used tree swallows (*Tachycineta bicolor*) to examine the possibility of transgenerational immunity and its effect on nestling growth and immune development. We measured cell-mediated immunity with a delayed-hypersensitivity assay and antibody-mediated immunity with a hemagglutination test with sheep red blood cells (SRBCs). We tested for differences in immunocompetence and growth between nestlings from females who had been exposed to a novel antigen prior to egg laying and nestlings from unexposed females. To determine whether the effect, if any, resulted from something transferred to the eggs prior to egg laying or from subsequent changes in parental behaviour, nestlings were exchanged so that at each nest half the nestlings were from females who had been injected with SRBCs and half were from females who had not been exposed to SRBCs. Finally, brood sizes were independently manipulated to either 4 or 6 nestlings. We failed to detect maternal antibodies in any nestlings, and whether a female was exposed to SRBCs or not had no effect on the growth or cell-mediated immunity of her brood. However, nestlings in smaller broods grew better than nestlings in larger broods, though we did not find the expected differences in cell-mediated immunity. Furthermore, within each nest, nestlings whose mothers had been exposed to SRBCs grew better than nestlings whose mothers had not been exposed. These results are contrary to the idea of a simple trade-off in the allocation of resources between parasite protection and reproduction; however, they support the idea that exposure of females to parasites prior to egg laying leads to better nestling growth, and are congruous with the possibility of mithridatic parental care.

**Résumé** : La présence d'un anticorps d'origine maternelle dans les oeufs des oiseaux est un fait bien connu, mais ce n'est que récemment que ce phénomène a été examiné dans un contexte écologique. Nous avons utilisé des hirondelles bicolores (*Tachycineta bicolor*) pour vérifier l'existence possible d'une immunité transmise d'une génération à l'autre et ses effets éventuels sur la croissance des oisillons au nid et sur le développement de leur système immunitaire. Nous avons mesuré l'immunité d'origine cellulaire au cours d'un test d'hypersensibilité retardée et l'immunité assurée par les anticorps au moyen d'un test d'hémagglutination SRBCs (globules rouges de mouton standardisés). Nous avons cherché à établir s'il y a des différences dans l'immunocompétence et la croissance chez les oisillons issus de femelles exposées à un antigène nouveau avant la ponte et chez les oisillons de femelles non exposées. Pour déterminer si cet effet, s'il existe, résulte d'un transfert aux oeufs avant la ponte ou des changements subséquents dans le comportement parental, les oisillons ont été déplacés de telle sorte que, dans chaque nid, la moitié des oisillons étaient issus de femelles qui avaient reçu une injection de SRBC et la moitié, issus de femelles non exposées aux SRBCs. Enfin, les couvées ont été fixées indépendamment à 4 ou 6 oisillons. Nous n'avons détecté d'anticorps maternel chez aucun des oisillons et l'exposition d'une femelle à des SRBCs est sans effet sur la croissance ou l'immunité d'origine cellulaire de sa progéniture. Cependant, les oisillons des couvées moins nombreuses se développent mieux que les oisillons des couvées plus grandes, mais nous n'avons pas observé les différences prévues quant à l'immunité d'origine cellulaire. De plus, dans chaque nid, les oisillons issus de mères exposées aux SRBCs ont eu une croissance meilleure que celle des oisillons issus de mères non exposées. Ces résultats contredisent l'hypothèse d'un simple compromis dans l'allocation des ressources entre la protection contre les parasites et la reproduction, mais ils sont en accord avec l'hypothèse selon laquelle l'exposition aux parasites avant la ponte donne lieu à une meilleure croissance des oisillons et ne contredisent pas la possibilité de soins parentaux mithridatiques.

[Traduit par la Rédaction]

## Introduction

Over the past decade, interest by ecologists in the immune system, perhaps initially precipitated by Folstad and Karter's (1992) immunocompetence-handicap hypothesis, has led to work in at least three directions. First, much recent work has been conducted under the mantle of the immunocompetence-handicap hypothesis (e.g., Ros et al. 1997; Hasselquist et al.

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1999; Duffy et al. 2000; Evans et al. 2000; Peters 2000; Poiani et al. 2000). Second, a change of emphasis has occurred in our methods; formerly, parasite load was used as an index of parasite resistance, but now we have started to examine immune condition directly. Finally, interest in the immune system has led researchers to examine potential trade-offs between maintenance of the immune system and several other fitness components (e.g., Richner et al. 1995; Deerenberg et al. 1997; Nordling et al. 1998; Moret and Schmnid-Hempel 2000; Norris and Evans 2000).

If we assume that the cost of immune defence is ecologically relevant, then the reallocation of resources becomes not only a theoretical expectation but also a physical necessity. When more than two factors are at play, however, the expected relationships are obfuscated, as, for example, in the interaction between the immune condition of parents, their reproductive choices, and development of their offspring. According to the trade-off view we would expect that an immune challenge just prior to a reproductive bout would have a negative impact on offspring. In contrast, the effect of the challenge and its accompanying immune response could be positive if offspring benefit from the immune responses of their parents. A well-documented mechanism by which these three factors may interact is the passage of maternal antibody during egg formation (Patterson et al. 1962; Williams 1962; Carroll and Stollar 1983), but only recently have the ecological implications begun to be examined.

By sterilizing great tit (*Parus major*) nests and later reinfesting some nests with fleas, Heeb et al. (1998) manipulated the level of flea infestation so that two groups of nests had different levels of flea infestation during egg laying but were similarly infested during incubation and brood rearing. Surprisingly, pairs that had been experimentally exposed to fleas during egg laying had fewer nest failures, larger and faster growing nestlings, greater fledging success, and more grandnestlings than unexposed pairs. These results are contrary to the idea of a simple resource-allocation trade-off between immune defence and reproduction. They could indicate increased parental effort by flea-exposed females, or transgenerational passage of acquired immunity at the time the eggs are laid. In this study we used tree swallows (*Tachycineta bicolor*) to examine these two possibilities more directly.

Specifically, we addressed the following questions: (*i*) does brood size affect nestling growth and immune function, (*ii*) are anti-sheep red blood cell (SRBC) antibodies transferred from mothers to offspring, and (*iii*) if there is a positive effect of maternal immune challenge on nestling development, is it because challenged females work harder at raising their broods or because something is transferred directly to the eggs at the time of egg laying. We exposed prelaying females to SRBCs and sampled their nestlings for the presence of SRBC-specific antibodies. To determine whether any effect on nestling development resulted from something that occurred at the time the eggs were laid or from subsequent changes in female behaviour, we exchanged hatchlings from nests where the female had been injected with SRBCs with hatchlings from nests where the female had not been injected. Hence, an effect on the entire brood would be evidence of changes in female behaviour, but differences between the two types of nestlings within each brood would necessarily be a result of factors that occured before hatch. Finally, this exchange was coupled with a brood-manipulation experiment to examine whether the stress of being part of a larger brood affected either measure of acquired immunity. We used a SRBC hemagglutination test (Wegmann and Smithies 1966; Aitken and Parry 1974; Klasing 1988; Deerenberg et al. 1997) and a subcutaneous delayed-hypersensitivity response (Corner and DeLoach 1990; Roitt et al. 1996) to assess humoral and cell-mediated acquired immunity, respectively.

## Methods

### General methods

The study was carried out from May to July of 1998 and 1999 at the Creston Valley Wildlife Management Area (49°07′N, 116°91′W) near Creston, British Columbia, Canada, where tree swallow boxes have been maintained for over a decade. The internal dimensions of these boxes are 10 × 10 × 30 cm, with a 3.5 cm diameter entry hole. Boxes are fixed on wooden or metal poles, with the bottom at approximately 1.5 m from the ground, and separated from each other by at least 15 m. All nesting material from previous years was removed before the start of the breeding season.

Starting in early May all nests were checked daily for the first signs of nest building. When a given nest was completed, the resident female was captured and banded. Because of age-related differences in reproductive performance (Lozano and Handford 1995), only after-second-year (ASY, adult, blue) females were used in the study and any second-year (1 year old, brown) females captured were simply banded and released. Every second ASY female captured was injected intraperitoneally with 0.05 mL of a suspension of washed SRBCs (Sigma Product No. R 3378) in phosphate-buffered saline (PBS, pH = 7.2, equivalent to $5 \times 10^8$ cells/mL, hematocrit ~2%). The mean interval between capture the start of egg laying did not vary significantly depending on whether or not the female was injected with SRBCs (11.5 vs. 10.2 days; ANOVA, $F_{[1,45]} = 0.646$, $p = 0.426$). Sham injections were not used because the response sought was specific to the antigen used, and if present, could have not been caused by the solution.

At hatch, nests at which females had been injected were paired with nests with the same hatch date at which females had not been injected. Out of each such pair one nest was randomly assigned to a brood size of 4 nestlings and the other one to a brood size of 6 nestlings. Nestlings were exchanged accordingly. Furthermore, nestlings were also distributed so that half the nestlings assigned to each nest came from females who had been injected with SRBCs and the rest from females who had not been exposed to SRBCs. Nestlings within each nest were individually marked and remarked as needed with a nontoxic marker on the underside of the wings and (or) legs. Brood size can be manipulated in two ways: (1) by changing the original brood size by a predetermined number of nestlings or a proportion of the original brood size, or (2) by setting the brood size to a predetermined number, irrespective of initial brood size. By necessity these two methods are mutually exclusive, except when the initial brood size is constant.

The result was a completely randomized 2 × 2 design, with female treatment (injected or not) and brood size (4 or 6) as the main (between-broods) effects and nestling treatment

(mother injected or not) as the nested (within-brood) effect. Hereinafter these factors will be referred to as female treatment, brood size, and nestling treatment, respectively. Female-treatment effects relate to the entire brood and hence reflect post-hatch effects of parental behaviour, whereas nestling-treatment effects occur within each brood, and hence relate to maternal effects that took place before hatching, presumably when the eggs were produced.

A 100-μL blood sample was taken from each 10-day-old nestling. At this age nestlings were also injected with SRBCs as described above, and banded with standard U.S. Fish and Wildlife Service (USFWS) leg bands. A second 100-μL blood sample was taken 6 days later. Blood samples were centrifuged to terminal hematocrit to separate the plasma, which was stored frozen at under –20°C until analysis. The body mass and wing-chord length of all nestlings were measured at 10 and 16 days after hatch.

### Delayed-hypersensitivity assay

At 15 days of age each nestling was given a hypodermic injection of 0.03 mL of phytohemagglutinin (PHA-P Sigma Product No. L8754) solution (2 mg of PHA/mL PBS) in the patagium of one wing, selected randomly. As a control each nestling was also given a 0.03-mL injection of only PBS in the patagium of the other wing. PHA attracts T-lymphocytes and induces them to proliferate, which results in a small swelling at the site of injection. The thickness of the PHA-injected and control patagia were measured 24 h post injection with a pressure-sensitive micrometer. The difference between the two measures indicates the strength of the response. Measurements obtained using this method are reasonably consistent (overall measurement error 12.1% (ANOVA); Lozano and Lank[3]).

### Hemagglutination assay

Using V-shaped 96-well microtiter plates, 25 μL of plasma from each sample was serially diluted with 25 μL of PBS, resulting in a twofold dilution series (i.e., $2^{-1}$, $2^{-2}$, $2^{-3}$, $2^{-4}$, …). Twenty-five microlitres of a suspension of 2% SRBCs in PBS was then added to each well. The plate was incubated at 41°C for 1 h and examined visually to determine the highest dilution (or lowest concentration) of plasma that led to an agglutination of SRBCs. Results are expressed as the negative power of the base-2 dilution factor (Wegmann and Smithies 1966; Aitken and Parry 1974).

### Statistical analyses

Between-brood and within-brood effects were analysed separately. Between-brood differences in size, mass, antibody production, and delayed-hypersensitivity response were analysed using the means for each nest in a fully factorial type III sums-of-squares 2 × 2 type I ANOVA, with female treatment (injected or not) and brood size (4 or 6) as the main effects. Nestling mortality was marginally higher at larger broods (continuity-adjusted $\chi^2 = 3.45$, $n = 47$, $p = 0.063$; Fienberg 1977) but was not associated with whether the female had been injected or not (continuity-adjusted $\chi^2 = 0.03$, $n = 44$, $p = 0.86$). Nests in which more than one nestling was lost were not included in any subsequent analyses.

The within-brood nestling treatment effect was assessed by comparing within each nest the mean values for nestlings whose mothers had been exposed to SRBCs with the mean values for nestlings whose mothers had not been exposed, using a Wilcoxon's matched-pairs signed-ranks test. All analyses followed the standard tests for homoscadasticity and homogeneity of variance when appropriate. Statistical significance was accepted at $p < 0.05$.

## Results

We were unable to detect any anti-SRBC antibodies in nestlings either at 10 days of age, or at 16 days of age, after they had been exposed directly.

Females assigned to specific treatments were not a biased subsample of the females that were initially pretreated (injected or not) several days before. Females assigned to the two treatments did not differ in mass (ANOVA, $F_{[1,45]} = 0.023$, $p = 0.8779$) or size (wing-chord length, $F_{[1,45]} = 0.007$, $p = 0.934$). Similarly, the time-span from the date of capture to the date the first egg was laid did not differ significantly between the two groups (ANOVA, $F_{[1,45]} = 0.646$, $p = 0.426$).

Female treatment did not have a significant effect on nestling mass (ANOVA, day 10: $F_{[1,45]} = 0.00006$, $p = 0.994$; day 16: $F_{[1,43]} = 0.1818$, $p = 0.672$), wing-chord length (ANOVA, day 10: $F_{[1,45]} = 0.0081$, $p = 0.9285$; day 16: $F_{[1,43]} = 0.101$, $p = 0.752$), or delayed-hypersensitivity response (ANOVA $F_{[1,41]} = 0.459$, $p = 0.501$). Therefore, whether or not a female was exposed to SRBCs before egg laying had no effect on her brood as a whole.

Nestlings in smaller broods grew better than nestlings in larger broods. Ten-day-old nestlings of smaller broods were significantly heavier but did not have longer wing chords than nestlings of larger broods (Table 1). Sixteen-day-old nestlings from broods with 4 nestlings were both larger and heavier than those from broods with 6 nestlings (Table 1). Cell-mediated immunity was not affected by brood size (Table 1).

Within broods, nestlings whose mothers had been exposed to SRBCs were generally larger and heavier than nestlings whose mothers had not been exposed. At 10 days of age, this nestling treatment effect was evident on both nestling mass (Fig. 1*a*) and wing-chord length (Fig. 1*b*). By 16 days of age significant differences in wing-chord length were still evident (Fig. 2*b*), but the effect on nestling mass had vanished (Fig. 2*a*).

## Discussion

### Responses to SRBCs

Contrary to the idea of a simple resource-allocation trade-off between offspring rearing and parasite protection, Heeb et al. (1998) found that in female great tits, parasite exposure during egg laying had an array of positive effects, including a lower rate of nest failure, higher hatching success, larger and faster growing nestlings, earlier fledging, and more grandnestlings. Heeb et al. (1998) suggested that these results could be explained if females developed antibodies to nest parasites and then transferred these antibodies to their

[3] G.A. Lozano and B.D. Lank. Imunocompetence and testosterone-dependent condition traits in male ruffs (*Philomachus pugnax*). Submitted for publication.

**Table 1.** Mass, size, and cell-mediated immunity of tree swallow (*Tachycineta bicolor*) nestlings in relation to brood size.

| | Four nestlings | | Six nestlings | | | |
|---|---|---|---|---|---|---|
| | *n* | Mean | *n* | Mean | *F* | *p* |
| Ten-day-old nestlings | | | | | | |
| Mass (g) | 24 | 20.9 (0.23) | 23 | 19.7 (0.37) | 8.84 | 0.005 |
| Wing-chord length (mm) | | 46.9 (0.59) | | 45.7 (0.79) | 1.12 | 0.296 |
| Sixteen-day-old nestlings | | | | | | |
| Mass (g) | 24 | 20.9 (0.18) | 21 | 19.62 (0.25) | 14.36 | <0.001 |
| Wing-chord length (mm) | | 78.8 (0.51) | | 76.4 (0.85) | 6.14 | 0.017 |
| Response to PHA (mm) | 22 | 0.396 (0.022) | 21 | 0.393 (0.214) | 0.014 | 0.904 |

**Note:** Values in parentheses show the standard error.

nestlings at the time of egg formation. Furthermore, Heeb et al. (1998) provocatively proposed that mothers purposely expose themselves to parasites prior to egg laying, in effect acting mithridatically[4], not for their own benefit but for that of their offspring. In this study we attempted to test and expand this idea and the alternative that challenged females work harder at raising their offspring. However, we failed to find any direct evidence of antibody transfer, or of SRBC-antibody production by nestlings that had been directly exposed.

Several factors could explain these results. An intriguing possibility is that some birds have the ability to shut off immune responses at times of strenuous activity, such as reproduction (Deerenberg et al. 1997). It has been argued that unless the invading pathogen is immediately life-threatening, the host could choose to ignore it and instead concentrate its resources on rearing its offspring (Forbes 1993); this tactic is most likely to be used by short-lived species with yearly breeding bouts and low overwintering survival. Second, the amount of maternal antibody in nestlings decreases sharply during the first few days after hatch (Apanius 1998), so it is possible that nestlings were sampled too long after hatch, when all traces of maternal antibody had disappeared. A response by nestlings directly challenged with SRBCs was probably lacking because at that early age their immune system is not yet well developed (Apanius 1998), which is the very reason why a transfer of maternal antibodies is advantageous. Finally, the amount of antibody transferred is highly variable (McIndoe and Culbert 1979; Smith et al. 1994), and the assay we used may not have been sensitive enough to detect the response.

SRBCs were originally employed in immunology because they could fortuitously be used to differentiate human T- and B-lymphocytes. They are still used in a variety of species because they contain a mixture of proteins, which usually ensures that at least some of these proteins are sufficiently antigenic. Although this assay has been used successfully with other species (Apanius 1998), it is not the most sensitive one available. Several workers have opted to quantify responses to single antigens via enzyme-linked immunosorbent assay (ELISA) (Ostrowski et al. 1989; Nordling et al. 1998; Hasselquist et al. 1999), and it has also been suggested that the immune system is best assessed with a multifaceted approach using several assays (Vos 1980; Luster et al. 1988; Weeks et al. 1992; Fairbrother 1994).

### Brood-size effects

Nestlings in smaller broods were larger than those in larger broods. This effect of brood size on nestling growth was as expected and is in agreement with the findings of many brood-manipulation studies (e.g., Hochachka and Smith 1991; Magrath 1991; Martins and Wright 1992; Markman et al. 1995; Lozano and Lemon 1998). These results are often presented as examples of the limitations of parental care or of a trade-off between offspring quality and quantity, and are further supported by the marginally higher nestling mortality in larger broods. Contrary to our expectations, cell-mediated immunity was not higher in nestlings from larger broods. Previous studies have shown decreased responses under experimentally induced nutritional stress (Glick et al. 1983; Klasing 1988; Lochmiller et al. 1993), but these food-restriction manipulations may not be analogous to the effects brought about by changes in brood size.

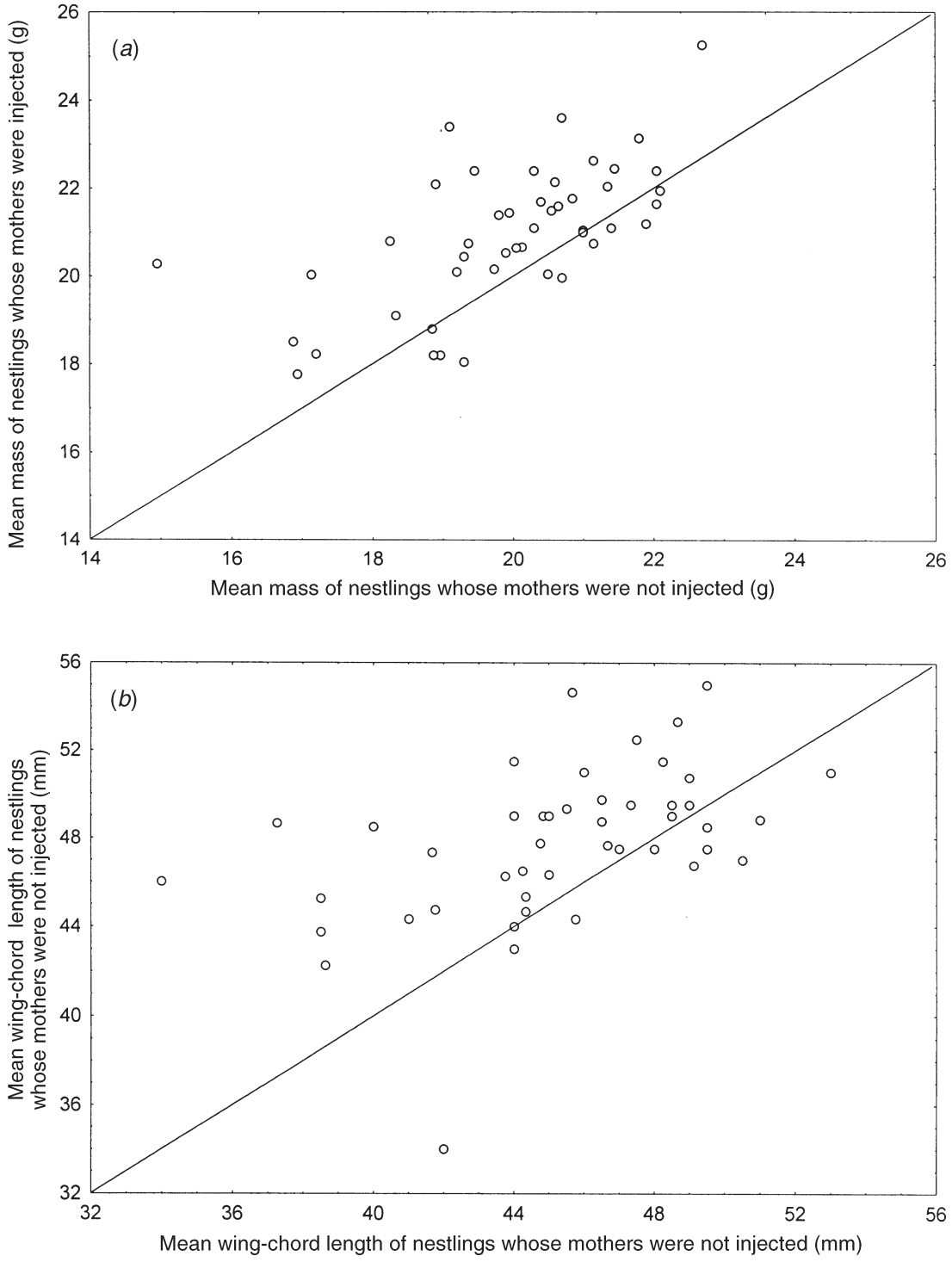
### Within-brood maternal effects on nestling growth

Perhaps the most fascinating result we obtained was that within each nest, irrespective of the between-nest treatment, tree swallow nestlings whose mothers had been exposed to SRBCs grew better than nestlings whose mothers had not been exposed. The effect was not present for mass at 16 days of age, but by that time wing-chord length is a better indicator of condition than mass, as the most developed nestlings within a brood may have already started the mass recession that precedes fledging (Zach and Mayoh 1982).

It is possible that nestlings whose mothers had been exposed to SRBCs were already different from their nestmates if treatment with SRBCs prior to egg laying led to differences in the females' likelihood of nesting and (or) in the delay from capture to egg laying. However, the mean wing-chord length, mass at initial capture, or time-span from capture to egg laying did not differ significantly between the two groups of females. A laboratory study designed specifically to test this possibility showed that, contrary to expectations, a SRBC challenge did not cause a reduction in clutch size, a longer delay to egg laying, or lower fledging success (Williams et al. 1999).

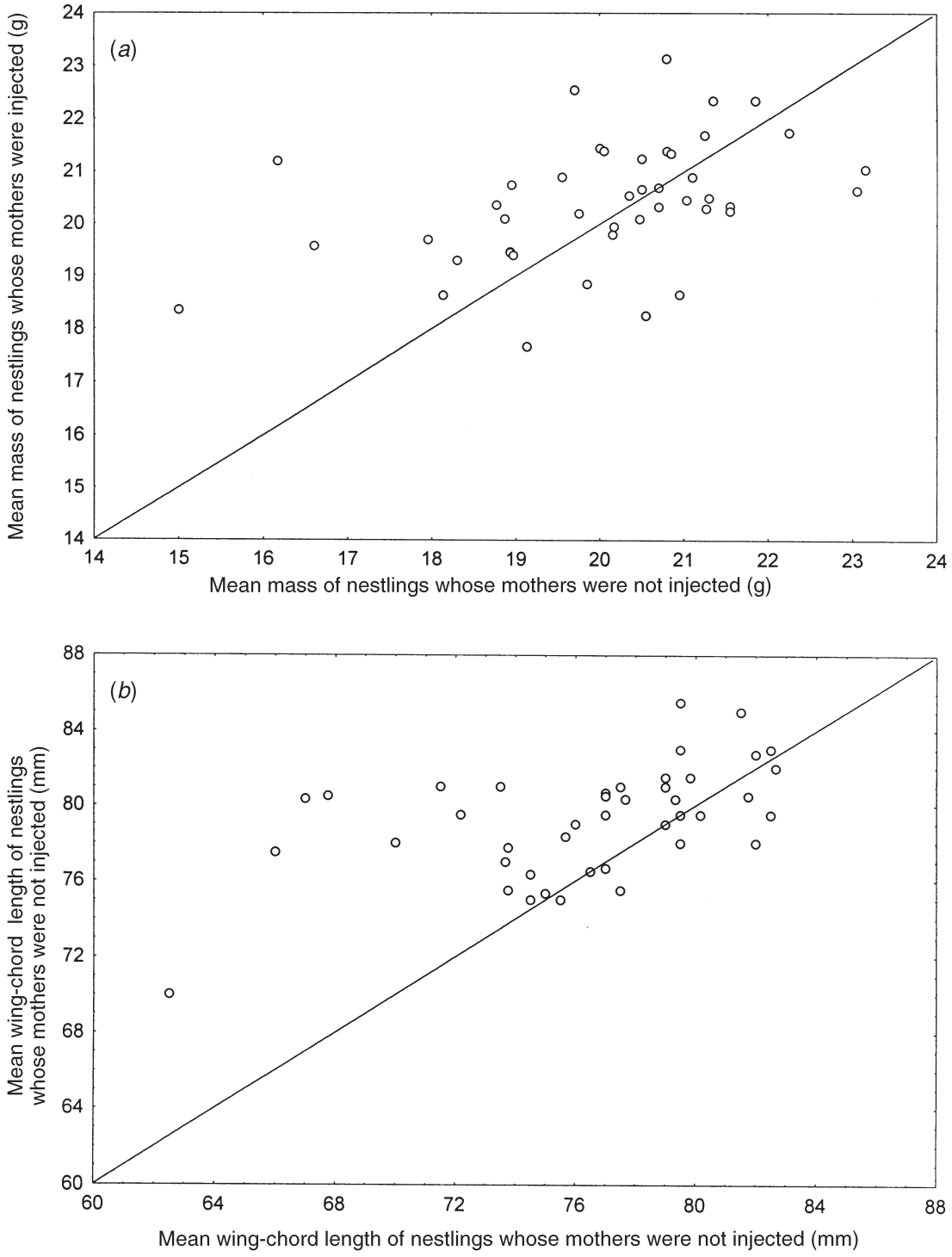
[4] After Mithridates VI (123–63 B.C.), King of Pontus, who, legend has it, purposely exposed himself to poisons in an attempt to become immune to them.

**Fig. 1.** Effect of maternal immune challenge on growth of tree swallow (*Tachycineta bicolor*) nestling at 10 days post hatch. Each point represents a nest. On the *x* axis is the mean value for nestlings within a nest whose mothers were not exposed to sheep red blood cells (SRBCs) and on the *y* axis the mean value for those nestlings within the same nest whose mothers were exposed to SRBCs. The solid line has a slope of 1, around which all points would fall if there were no effect of nestling treatment on growth. (*a*) Body mass; Wilcoxon's matched-pairs signed-ranks test, $T = 164$, $Z = 4.1$, $p < 0.0001$, $n = 47$. (*b*) Wing-chord length; $T = 166$, $Z = 4.09$, $p < 0.0001$, $n = 47$.




Heeb et al. (1998) showed that great tit nestlings whose mothers had been exposed to fleas actually performed better during the nestling period, the interpretation being that flea-exposed mothers developed immunity against fleas and passed it on to their nestlings. In our experiment we were unable to detect a transfer of antibodies directly, but the experimental design eliminated several alternative explanations. First, the effect occurred within nests so it was not the result of in-

**Fig. 2.** Effect of maternal immune challenge on nestling growth at 16 days post hatch. (*a*) Body mass; Wilcoxon's matched-pairs signed-ranks test, $T = 348$, $Z = 1.503$, $p = 0.1327$, $n = 44$. (*b*) Wing-chord length; $T = 108$, $Z = 4.05$, $p < 0.0001$, $n = 44$.




creased parental effort, which would have been possible if females perceived an immune challenge as an increase in the risk of mortality and consequently increased their reproductive effort. Second, by using a nonpathogenic antigen instead of a parasite we avoided the possibility that the parental immune responses were detrimental to the parasites (Wikel et al. 1996; Jones 1996; Ghosh and Mukhopadhayay 1998) and only indirectly led to better nestling growth. The fact that we used a nonpathogenic antigen instead of a specific parasite also suggests a more general explanation: that any activation of a mother's immune system, presumably within a reasonable range, may actually lead to better nestling growth.

Therefore, the effect and general route are clear: a maternal immune challenge had a positive effect on nestling growth, and was due to something that occurred at the time the eggs were produced, not thereafter. We can envision two potentially interacting mechanisms. First, it is possible that an immune challenge actually caused females to transfer to their

eggs something other than immunological products; recent work has demonstrated that birds can differentially allocate testosterone to their eggs, which in turn affects intrabrood competitiveness (Schwabl et al. 1997; Lipar and Ketterson 2000). Second, nestlings may have been bequeathed an advantage against parasites when their mother's immune system was activated and antibodies were deposited in the developing egg (Patterson et al. 1962; Williams 1962; Carroll and Stollar 1983; Gasparini et al. 2001). It will be necessary to detect what exactly is being transferred in order to determine whether the mechanism is hormonal or immunological, but either scenario opens interesting possibilities. The former points to parental manipulation of offspring competitiveness and the latter to mithridatic parental care.

**Conclusion**

Much effort is currently being devoted to assessing pairwise trade-offs between immune responses and other fitness components. Several studies have shown some of the expected relationships (e.g., Deerenberg et al. 1997; Nordling et al. 1998; Ilmonen et al. 2000) and others have failed to do so (Nordling et al. 1998; Hasselquist et al. 1999; Moreno et al. 1999; Williams et al. 1999), which prompted Williams et al. (1999) to speculate that the costs of mounting an immune response are, if not negligible, at least ecologically irrelevant under the conditions usually tested. Although it is understandably difficult, if not impossible, to determine the exact cost, energetic or otherwise, of maintaining the immune system or responding to a specific challenge, the resources allocated to these functions are likely proportional to the expected fitness losses brought about by parasites. This argument does not simply replace an immeasurable with an intangible. Given the sheer abundance and ubiquity of parasites (Windson 1998), the expected fitness losses are considerable, as illustrated by the way in which immunocompromised animals are quickly decimated by parasites, so the effort allocated to parasite protection must be equally substantial. As shown in this study, however, other factors may be at play, which may hinder our ability to detect these pairwise trade-offs. Although there is certainly some utilitarian value in the pairwise trade-off approach, it is perhaps time we expanded our paradigm to acknowledge that the immune system is involved in multifaceted interactions.

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