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Age at weaning, immunocompetence and ectoparasite performance in a precocial desert rodent

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ABSTRACT

We studied the effects of early weaning on immunocompetence and parasite resistance in a precocial rodent *Acomys cahirinus*. We hypothesized that if parasite resistance is energetically expensive and nutritional and immunological support from mothers are necessary for the long-term health of offspring, then early weaned animals would be immunologically weaker and less able to defend themselves against parasites than later weaned animals. We weaned pups at 14, 21 or 28 days after birth and assessed their immunocompetence and resistance against fleas *Parapulex chephrenis* when they attained adulthood. Immunocompetence was assessed using leukocyte concentration (LC) and a phytohaemagglutinin injection assay (PHA test). To estimate resistance against fleas, we measured performance of fleas via the number of produced eggs and duration of development and resistance to starvation of the flea offspring. We found a significant positive effect of weaning age on the PHA response but not on LC. The effect of age at weaning on flea egg production was manifested in male but not female hosts, with egg production being higher if a host was weaned at 14 than at 28 days. Weaning age of the host did not affect either duration of development or resistance to starvation of fleas produced by mothers fed on these hosts. We conclude that even in relatively precocial mammals, weaning age is an important indicator of future immunological responses and the ability of an animal to resist parasite infestations. Hosts weaned at an earlier age make easier, less-resistant targets for parasite infestations than hosts weaned later in life.

KEY WORDS: Fleas, PHA response, Weaning, Spiny mouse

INTRODUCTION

It is well known that resistance against parasites is often energetically expensive and a number of studies have investigated the potential trade-offs between resistance and other energetically demanding processes (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Rigby et al., 2002; Sandland and Minchella, 2003; but see Klasing, 1998; Hasselquist and Nilsson, 2012). This

trade-off may be particularly noticeable in energy-limited organisms, or young animals facing elevated energetic demands associated with growth (Ricklefs and Wikelski, 2002; Krasnov et al., 2005). While the exact cost of resistance remains difficult to quantify, it is increasingly clear that from a life-history perspective, maintenance and upregulation of immune function may suffer in the face of a limited energy supply (Lochmiller and Deerenberg, 2000; Pilorz et al., 2005; French et al., 2009).

Reproductive choices concerning weaning time in mammals are likely to have a great effect on a number of life-history parameters. Evolutionarily, mammalian mothers face a conflict: lactation is well-known to be energetically demanding and it delays future reproductive events; however, early weaning may place nursing offspring at greater risk for restricted growth or increased susceptibility to pathogens or parasites (Grindstaff et al., 2003; Grindstaff, 2008). Mothers absorb some of the energetic costs placed on their young through lactation. In addition to the strict energetic value of lactation for offspring, mothers also transfer immunologically valuable antibodies prenatally through the placenta and postnatally in colostrum and milk. The time for which maternal antibodies persist in the offspring differs depending on the species and the time required for offspring to begin their own significant endogenous antibody production (Grindstaff et al., 2003; Grindstaff, 2008). Early weaning may be expected to increase the energetic burden placed directly on the young offspring and therefore limit the energy allocated toward other competing challenges. Consequently, offspring that are weaned early may be less immunologically prepared and, in essence, have a smaller arsenal of tools with which to resist parasites and pathogens.

Detrimental effects of early weaning have been seen in a number of mammals – especially those important to the livestock industry. Young ruminants, particularly cattle and sheep, are more susceptible to infections and increased parasite burdens seemingly due to elevated glucocorticoids, immunosuppression and overall decreased immune responsiveness (Watson and Gill, 1991a; Watson and Gill, 1991b; Colditz et al., 1996). Early weaning is associated with increased stress hormone levels, a lower growth rate and overall immunosuppressive effects in sheep (Napolitano et al., 2008). Early weaning stress in pigs has been linked with chronic activation of the hypothalamic-pituitary–adrenal axis and increased cortisol levels, and disruption in normal intestinal function (Smith et al., 2010).

Although most studies of the effect of weaning age on parasite resistance have focused on precocial animals (primarily because altricial animals are not often able to survive when they are weaned too early), there are also a number of studies using early maternal separation in rats as a model of early life trauma (Slotten et al., 2006; Gareau et al., 2007). The severity of the effects of maternal separation varies; however, rat pups that are subjected to maternal separation tended to show immediate and long-term alterations in behavioural patterns as well as hormonal and immunological

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profiles that may contribute to decreased resistance to diseases, parasites and pathogens later in life (McCormick et al., 1998; Slotten et al., 2006; Gareau et al., 2007).

Although the effects of early weaning on parasite resistance have been well documented in livestock, to our knowledge they have not been studied in small wild mammals. In part, this void exists because there are relatively few precocial small wild mammals available with which to successfully manipulate weaning age without significantly risking survival of the animals. In the present study, we were interested in the effects of early weaning on immunocompetence and parasite resistance in a precocial small mammal. We used *Acomys cahirinus* (Desmarest) and its specific flea *Parapulex chephrenis* (Rothschild) as a model host–parasite association. *Acomys cahirinus* is a small mammal (adults typically weigh 40–60 g) with a relatively long gestation period for its size (~37–41 days). They are able to produce two to three litters per year (one to four pups per litter). This species is considered precocial because pups are born with developed senses and fur cover, start to eat solid food 2–3 days after birth and are naturally weaned at 2–3 weeks (Yarmolinsky et al., 2009). Fleas (Siphonaptera) are haematophagous holometabolous insect parasites commonly found on small mammals. They alternate periods when they occur on the host's body and when they reside in the host's burrow or nest. Although *P. chephrenis* spends most of its time in the host's fur, its pre-imaginal development (eggs, larvae and pupae) occurs solely off-host (Krasnov, 2008). Fleas represent a convenient model for studies of various aspects of host–parasite interactions because, in contrast to many other parasite taxa, it is easy to manipulate flea infestation on living hosts both in the field and in the laboratory and to monitor changes in both hosts and fleas over time. In the central Negev desert, *A. cahirinus* and its congeneric *Acomys russatus* are by far the most common host for *P. chephrenis*, and *P. chephrenis* does not typically parasitize other host species.

We hypothesized that if parasite resistance is energetically expensive, and nutritional as well as immunological support from mothers is necessary for the long-term health of offspring, then animals that are weaned early should be immunologically weaker and therefore less able to defend themselves against parasite infestations. To test this hypothesis, we weaned pups of *A. cahirinus* at 14, 21 or 28 days after birth and assessed their immunocompetence and resistance against fleas when they attained adulthood (at 65 days after birth). Immunocompetence was evaluated using immunological parameters linked with cell-mediated immunity. We chose to measure cell-mediated rather than humoral immunity parameters because earlier studies have shown that, in rodents, the immune response to flea parasitism is linked mainly to cell-mediated immunity (Khokhlova et al., 2004a; Khokhlova et al., 2004b) [see also Rubaire-Akiki and Mutinga

(Rubaire-Akiki and Mutinga, 1980) and Heller-Haupt et al. (Heller-Haupt et al., 1996) for the role of cell-mediated rather than humoral immunity against ticks]. We assessed immunocompetence via one non-functional (leukocyte concentration, LC) and one functional (a phytohaemagglutinin injection assay, subsequently referred to as PHA test) technique. Both these measures are commonly used in ecoimmunological studies (e.g. Smits et al., 1999; Goüy de Bellocq et al., 2006a; Goüy de Bellocq et al., 2006b; Demas et al., 2011) and have become popular because they do not need sophisticated equipment. LC represents a measure of immune-related structure (e.g. Goüy de Bellocq et al., 2006a). Obviously, an individual with a relatively high LC is likely to possess better immune defences compared with an individual with a low LC.

The PHA test involves challenging an individual animal with antigens that trigger cellular immune response. The test is performed by subcutaneous injection of a vegetal lectin (PHA extracted from the red kidney bean *Phaseolus vulgaris*) that induces local T-cell stimulation and proliferation, resulting in swelling. The degree of swelling is considered as a measure of the potential proliferative response of circulating T-cells. The response to PHA is seen soon after injection (Smits et al., 1999). We predicted that rodents that are weaned early would have a less robust response when presented with an immune challenge. In other words, we predicted that both the PHA assay and LC would be positively correlated with weaning age.

To gain an understanding of the ecological relevance of host resistance by gauging the effect of resistance on parasite fitness (Combes, 2001), we measured the performance of fleas exploiting rodents weaned at different ages via the number of eggs produced by the fleas as well as the duration of development and resistance to starvation of the offspring of these fleas. If a host is more energetically or immunologically challenged and, consequently, less able to defend itself, we expected that fleas would produce more eggs when exploiting a host that is weaned early, and fleas of a new generation that hatch from these eggs would develop faster and survive longer under starvation (indicating a higher quality flea) (see Krasnov, 2008).

RESULTS

The results of mixed-effects models of PHA response and LC as affected by rodent sex and age at weaning are presented in Table 1. PHA response was significantly affected by age of weaning but not by sex or the interaction between the two factors. The model differed significantly from the intercept-only model (log-likelihood ratio=14.53, *P*=0.01). Furthermore, PHA response in rodents weaned at 28 days was significantly stronger than in those weaned at 14 or 21 days (Tukey's HSD tests, *z*=3.18 and *z*=2.51, respectively; *P*<0.05 for both), although it did not differ between

Table 1. ANOVA for linear mixed-effects models of phytohaemagglutinin response and leukocyte concentration as affected by rodent sex and age at weaning controlled for individual variation among rodent mothers

Dependent variable	Effect	d.f.	*F*	*P*
PHA	Intercept	1,99	2175.04	<0.001
	Sex	1,99	0.09	0.76
	Age at weaning	2,99	6.49	0.002
	Sex × age at weaning	2,99	0.81	0.47
LC	Intercept	1,99	85,145.61	<0.001
	Sex	1,99	1.78	0.18
	Age at weaning	2,99	2.29	0.11
	Sex × age at weaning	2,99	1.55	0.22

PHA, phytohaemagglutinin; LC, leukocyte concentration.
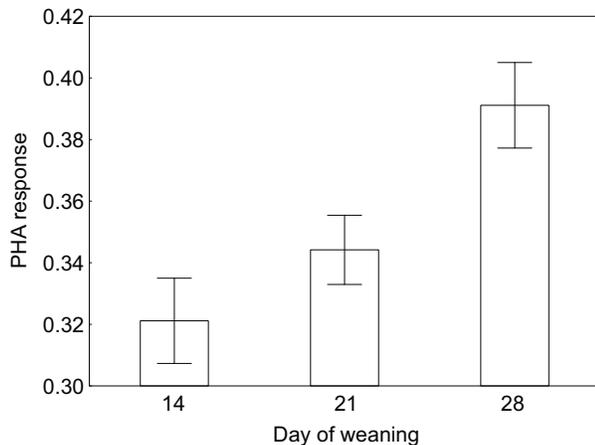Rodents were weaned at 14, 21 and 28 days.

Fig. 1. The relationship between phytohaemagglutinin (PHA) response and age at weaning. Mean (±s.e.m.) swelling response (PHA response) of *Acomys cahirinus* weaned at 14, 21 or 28 days and injected with a vegetal lectin (PHA) at the age of 65 days.
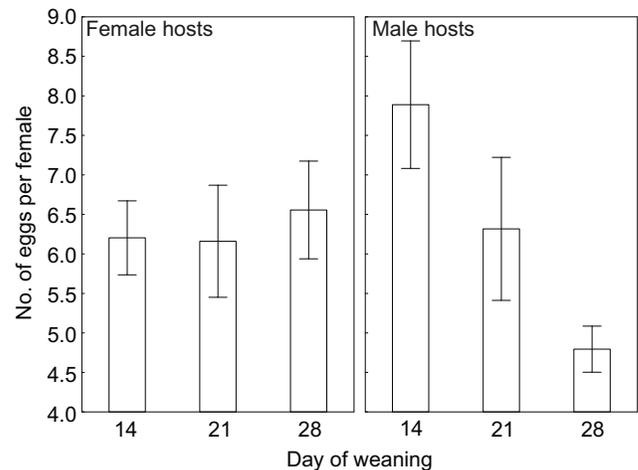

Fig. 2. The relationship between host age at weaning and flea egg production. Mean (±s.e.m.) number of eggs produced per female *Parapulex chephrenis* flea during 4 days of oviposition after feeding on 65 day old male and female *A. cahirinus* that were weaned at 14, 21 or 28 days.

rodents weaned at 14 and 21 days (Tukey's HSD test, z=1.09, P=0.52) (Fig. 1). In contrast, no effect of either factor or their interactions on LC was found. The model did not differ from the intercept-only model (log-likelihood ratio=9.6, P=0.09).

The results of mixed-effects models of flea egg production and two variables of flea offspring quality (duration of development and time to death under starvation) in response to host and/or flea sex and host age at weaning are presented in Table 2. All three flea models differed significantly from the respective intercept-only models (log-likelihood ratio=14.01, log-likelihood ratio=108.63 and log-likelihood ratio=24.41, respectively; P<0.01 for all). We found no independent effect of either host sex or age at weaning on the average number of eggs produced by a female flea during 4 days of oviposition (Table 2). However, the interaction between these two factors was significant. This was because the effect of age at

weaning on flea egg production was manifested in male but not female hosts (Fig. 2). Fleas exploiting male rodents weaned at 28 days produced significantly fewer eggs than fleas exploiting male rodents weaned at 14 days (Tukey's HSD test, z=−3.21, P=0.01; Fig. 2). However, no significant differences in egg production were found between fleas feeding on males weaned at 14 and at 21 days of age or at 21 and 28 days of age (Tukey's HSD tests, z=−1.20 and z=−1.30; P>0.77 for both; Fig. 2).

Age of weaning of a rodent host did not affect either duration of development or time to death under starvation of fleas produced by mothers fed on these hosts (Table 2). Duration of development was affected only by flea sex, with females developing faster than males (43.23±0.26 versus 46.00±0.31 days, respectively), while time to death under starvation was affected only by host sex, with fleas produced by mothers fed on male hosts surviving for longer

Table 2. ANOVA for linear mixed-effects models of flea egg production, duration of development and time to death under starvation of new generation fleas as affected by rodent sex and age at weaning controlled for individual variation among rodents and among their mothers

Dependent variable	Effect	d.f.	F	P
EP	Intercept	1,14	1103.35	<0.001
	Host sex	1,9	0.13	0.71
	Age at weaning	2,14	1.50	0.26
	Host sex × age at weaning	2,9	5.61	0.02
DD	Intercept	1,1241	35,044.61	<0.001
	Host sex	1,13	0.01	0.97
	Flea sex	1,1241	99.55	<0.001
	Age at weaning	2,14	0.05	0.95
	Host sex × flea sex	1,1254	0.13	0.71
	Host sex × age at weaning	2,13	2.54	0.11
	Flea sex × age at weaning	2,1241	2.85	0.09
	Host sex × flea sex× age at weaning	2,1241	0.45	0.64
DS	Intercept	1,1241	21,029.66	<0.001
	Host sex	1,13	7.93	0.01
	Flea sex	1,1241	1.29	0.26
	Age at weaning	2,14	1.48	0.26
	Host sex × flea sex	1,1254	3.29	0.07
	Host sex × age at weaning	2,13	1.96	0.18
	Flea sex × age at weaning	2,1241	2.99	0.06
	Host sex × flea sex× age at weaning	2,1241	0.93	0.39

EP, egg production (average number of eggs produced per female flea during 4 days of oviposition); DD, duration of development; DS, time to death under starvation.
The effects of sex of the flea's rodent host are only shown for DD and DS. Rodents were weaned at 14, 21 and 28 days.

(9.69±0.11 days for male hosts and 8.91±0.12 days for female hosts).

DISCUSSION

The results of this study partly supported our prediction. In general, rodents separated from their mothers later demonstrated stronger immunocompetence at maturity than those separated earlier, although this was manifested in the PHA response but not in LC. Flea parasites performed better on earlier than on later weaned rodents. Although this pattern was found in male but not female hosts and in the quantity but not the quality of parasite offspring, it definitely points out the negative relationship between the age of weaning and resistance against parasites. It should be noted that in our earlier study (Goüy de Bellocq et al., 2006a) we did not find a relationship between the PHA response of a rodent host and egg production of fleas exploiting it. However, that study (Goüy de Bellocq et al., 2006a) was carried out on different rodent host and flea species from those in the present study (*Meriones crassus* and *Xenopsylla ramesis*, respectively). In addition, other studies reported a relationship between the PHA response and host resistance against parasites (Gonzalez et al., 1999; Christe et al., 2000; Navarro et al., 2003). We recognize that different rodent individuals were used for immunocompetence and for flea performance measurements in this study (see rationale below). Consequently, the relationship between weak immune response and lower flea resistance was not tested directly. However, our results strongly, albeit indirectly, indicate the association between the relative weaker immunocompetence and better performance of parasites in earlier weaned rodents.

An earlier study of the effect of maternal deprivation in *A. cahirinus* (Yarmolinsky et al., 2009) demonstrated that pups weaned at 14 or 21 days lost body mass at the first post-weaning day, suggesting that they were still dependent on their mother's milk (Yarmolinsky et al., 2009). In contrast, animals weaned at 28 days did not decrease body mass after weaning. This was because they were already independent of milk and able to consume enough solid food to satisfy their energy requirements (Yarmolinsky et al., 2009). However, rodents weaned at different ages attained similar body mass at 64 days, suggesting that compensatory growth occurred in pups weaned at 14 and 21 days. Compensatory growth represents a period of accelerated growth following a period of slow growth or even decreasing body mass (associated with a nutritional deficit). Growth compensation allows animals exposed to a nutritional deficit early in life to reach definitive adult size. However, compensatory growth entails costs that may detrimentally affect their performance as adults (Metcalfe and Monaghan, 2001; Monaghan, 2008) in terms of, for example, survival and reproduction. For example, zebra finches reared on a low protein diet had shorter adult lives (Birkhead et al., 1999) and produced their first clutch later than finches on a high protein diet (Blount et al., 2006). Our results suggest that another cost of compensatory growth may be lower resistance to parasites because fleas produced twice as many eggs when they exploited male rodents weaned at 14 days as opposed to 28 days.

The negative long-term effects of compensatory growth may underlie the proposal for threshold pup mass as a proximate cue for the weaning decision, which was suggested to be based on both maternal and pup state (Lee et al., 1991). That hypothesis was not supported for extremely precocial species such as the guinea pig. Rehling and Trillmich demonstrated that the decision on the timing of weaning in guinea pigs appeared to be a solely maternal prerogative and did not depend on pup body mass but rather, presumably, on maternal milk yield (Rehling and Trillmich, 2007). This is because guinea pig pups provision themselves even at early stages of lactation and become independent of maternal milk before lactation is terminated (Künkele and Trillmich, 1997). However, *A. cahirinus* is characterized by a substantially lower degree of precociality than the guinea pig. Although pups of *A. cahirinus* are definitely precocial from a morphological point of view (born with developed senses and hair cover, are able to move starting the second day post-parturition and are able to consume solid food at 2–3 days), they seem to be altricial from a nutritional point of view because they require extended support from mothers (Yarmolinsky et al., 2009).

The negative impact of early weaning on various components of immunity has been repeatedly demonstrated for various livestock species (e.g. Blecha et al., 1983; Hulbert et al., 2011; McLamb et al., 2013; but see Kick et al., 2012). As a result of a decreased immune function, early weaned individuals exhibited an increase in parasite burden (Iposu et al., 2008; Iposu et al., 2010). For example, lambs allowed to suckle until 84–140 days and artificially infested with a nematode *Teladorsagia circumcincta* had a lower faecal egg count and worm burden at slaughter than their twins weaned at 39 days (Iposu et al., 2010). Our study supports these results because earlier weaned rodents demonstrated both a weaker PHA response and a higher egg production of flea parasites. The mechanism behind this pattern might be associated with energy allocation by growing pups.

It is commonly accepted that immunity is an energetically demanding process and one of the main reasons for immunosuppression is resource reallocation to other metabolic needs (Sheldon and Verhulst, 1996; Demas and Nelson, 1998; Ilmonen et al., 2003; but see Hasselquist and Nilsson, 2012). For example, Krasnov et al. demonstrated that fleas produced more eggs when they exploited malnourished rather than well-fed gerbils *Meriones crassus* (Krasnov et al., 2005). In our study, early weaned *A. cahirinus* were undoubtedly energy challenged. They not only had to compensate for the lack of maternal resources but also were forced to expend more energy for thermoregulation because, in contrast to later weaned individuals, they could not huddle with their mothers and siblings. As a result, they probably invested less energy in the development of immunity, which then impaired their immunocompetence at adulthood. An additional factor that might impair immunocompetence of early weaned *A. cahirinus* is the level of androgens such as testosterone that could suppress the immune function (Folstad and Karter, 1992; Zuk, 1996; Zuk and McKean, 1996). Although the relationship between age of weaning and the level of sexual hormones is poorly studied, some evidence suggests that early weaning may cause elevated testosterone. For example, plasma testosterone concentration in Angus bulls aged 263–375 days was higher in animals weaned at 120 days than at 207 days (Sitarz et al., 1977). The effect of weaning age on nutritional characteristics of the blood (e.g. Quigley et al., 1991) may also be responsible for differential flea responses when feeding on earlier versus later weaned mice. Although we measured flea performance in mice aged 65 days, so they could compensate for early weaning (see Yarmolinsky et al., 2009), the effects of early weaning on blood composition may be long lasting (Hahn and Kirby, 1973).

Although the effect of weaning age on immunocompetence as measured via the PHA response was similar in male and female *A. cahirinus*, its effect on flea performance was found in males only. This suggests that the interplay between energy availability during growth, morphophysiological development and development of immunity differs between males and females. This interplay may be affected by, for example, sex-specific susceptibility to early weaning stress. Indeed, Kikusui and Mori reported that male rodents are more

vulnerable to early weaning stress than females (Kikusui and Mori, 2009). Sexual difference in vulnerability to early weaning stress could be related to hormonal differences. Indeed, basal corticosterone levels were shown to be higher in early weaned than in normally weaned male laboratory mice, but this was not the case for females (Kikusui et al., 2006). Given that prolonged high glucocorticoid concentrations may lead to suppression of skin immune function (Dhabhar and McEwen, 1999) and immune function in general (Boonstra, 2013), the difference between males and females may explain, at least partly, why the effect of the age of weaning on flea egg production was manifested in male and not female *A. cahirinus*. The above-mentioned elevation of testosterone associated with early weaning (Sitarz et al., 1977) might also further suppress immune function in males but not in females. An alternative explanation for sex differences in the effect of weaning age on flea performance but not the PHA response may be associated with the fact that early weaning causes increased anxiety behaviour in male but not female rodents (Kanari et al., 2005; Ito et al., 2006; Kikusui et al., 2006). Fleas feed better on active/agitated than on confined/non-agitated hosts (Bar-Zeev and Sternberg, 1962; Suter, 1964; Jun et al., 1993) perhaps because of an increased peripheral blood supply (Suter, 1964). The improved feeding probably leads to higher egg production (for a review, see Krasnov, 2008). In other words, the mechanism of the effect of host weaning age on ectoparasite performance may not be immunological, so that male and female rodents of different weaning age in this study were similar in their immunocompetence characteristic but fleas performed differently on them.

Among the immunological variables measured in our study, we found an effect of age at weaning on the PHA response only, whereas there was no effect on LC. No relationship between these two variables was found in an earlier study on another rodent species, *M. crassus* (Goüy de Bellocq et al., 2006a). This suggests independence of these two measures of immune defence (Hõrak et al., 1999; Goüy de Bellocq et al., 2006a). Furthermore, the concentration of leukocytes and their activity are not necessarily correlated (e.g. Khokhlova et al., 2004b), so that the former variable might not reflect immunocompetence of our experimental rodents.

The results of this study suggest that host immunological and energetic preparedness early in life can have long-term implications in terms of parasite resistance. We conclude that even in relatively precocial mammals, weaning age is an important indicator of future immunological responses and the ability of an animal to resist parasite infestations. From the parasite's perspective, it is obvious that hosts weaned at an earlier age (i.e. more energetically or immunologically challenged) make more vulnerable targets for parasite infestations later in life.

MATERIALS AND METHODS
Rodents and fleas
We obtained rodents and fleas from our laboratory colonies started in 1999 from field-collected specimens. Rodents were maintained in plastic cages (60×50 cm and 40 cm high) with sawdust bedding at 25°C ambient temperature and 12 h dark:12 h light regime. Millet seeds and alfalfa (*Medicago* sp.) were offered *ad libitum*. No water was available to the rodents as the alfalfa supplied enough water for their needs. Rodents were offered commercial cat chow once a week. Initially, each cage contained a male and a female. Young individuals were moved to a new cage at 2 months of age to prevent overpopulation and inbreeding. In this study, for newly formed pairs we used unrelated rodents ca. 120 days old.

Fleas (*P. chephrenis*) were reared on *A. cahirinus*, using procedures described elsewhere (e.g. Sarfati et al., 2005; Khokhlova et al., 2008). An individual rodent host and its fleas were kept in a plastic cage (60×50 cm

and 40 cm high) with a wire mesh floor and a pan containing a mixture of sand and dried bovine blood (larvae nutrient medium) underneath. Once every 2 weeks, cages were cleaned and fleas were added back to the rodent host. Substrate and bedding material from the nestbox were collected, and transferred to an incubator (FOC225E, Velp Scientifica srl, Milan, Italy), where fleas developed at an air temperature of 25°C and relative humidity of 75%. In this study, we used only newly emerged fleas, 1–2 days old, which did not feed from emergence until experimental treatments.

Experimental design
To obtain animals weaned at different ages, we followed the protocol described previously (Yarmolinsky et al., 2009). Prior to experiments, 87 females were maintained individually in plastic cages as described above. They were mated by placing a male into the cage for 2 weeks. If a female was not pregnant, a male was placed in her cage again as above. In total, these females gave birth to 136 pups with litter size ranging from one to three pups. Within litter sizes, mothers were assigned randomly to one of the treatments in which pups were weaned at 14, 21 or 28 days. Each of these treatments contained six to eight females nursing one pup, 13–18 females nursing two pups and five to six females nursing three pups. Weaned pups were kept individually as described above. When pups reached the age of 65 days, we randomly selected 28–43 individuals from each weaning treatment and used them for immunocompetence measurements (PHA and LC; see below)]. The remaining 29 rodents (seven to 12 individuals per treatment) were used to study flea performance (see below). We used different animals for immunological and flea performance measurements because the PHA injection could affect the subsequent immune responses of an animal (Johnsen and Zuk, 1999; Goüy de Bellocq et al., 2006a). In fact, when a host is infected by two parasites simultaneously, one parasite affects the host responses to the second parasite through depression or stimulation of the immune response (Cox, 2001). As the host mounts an immune reaction against PHA, one might expect that these PHA-challenged individuals would mount a different response against fleas than individuals that had never been challenged by an antigen (e.g. PHA). Consequently, if the same individual is subjected to both immunological and parasitological measurements, the earlier tests for the PHA response could confound the results of the later tests on flea performance. Furthermore, given that the animals used in our experiments originated from the same ancestors, were maintained under the same conditions and were randomly assigned to either immunological or parasitological treatment, their defence against fleas was probably similar. The possible confounding effect of maternal identity was controlled for in the data analyses (see below).

Immunocompetence measurements
To measure, LC, we collected a blood sample from the infraorbital sinus of an animal using heparinized capillary tubes. The animals recovered fully within 2 min of blood sampling. Then, we diluted 4 µl of the whole blood with Türk solution (1:25) to ensure erythrocyte lysis, counted leukocytes under a light microscope at 40× magnification using a Bürker–Türk haemocytometer, and expressed concentration as number of cells mm^{-3}.

For the PHA test, we measured footpad thickness of the left hindfoot of a rodent. Then we injected subcutaneously 0.1 mg of PHA (PHA-P, Sigma L-8754, Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.03 ml of saline in the middle of the footpad, and measured footpad thickness 6 h post-injection. Our earlier results demonstrated that the PHA response in *A. cahirinus* is significantly higher at 6 h post-injection than at 24 or 48 h post-injection (Goüy de Bellocq et al., 2006b). The PHA response was calculated as the difference between pre- and post-injection measurements standardized for initial footpad thickness as: PHA response=(post-PHA–pre-PHA)/pre-PHA. We measured footpad thickness with a micrometer (Mitutoyo, type 547-301, Mitutoyo Corporation, Kawasaki, Japan) to ±0.01 mm. As the micrometer exerts a constant pressure on the footpad, to standardize the measurements we took readings 3 s after its application.

LC measurements were made in duplicate, whereas the PHA response measurements were made in triplicate to test the repeatability of measurements. Repeatability was calculated as the intraclass correlation coefficient based on variance components derived from a one-way ANOVA

(Lessells and Boag, 1987). Repeatability was high (r=0.85 for LC and r=0.99 for PHA response; P<0.0001 for all), so we used averaged values of both measurements in the subsequent analyses.

Flea performance measurements

We estimated flea performance via the quantity of offspring (the number of produced eggs) as well as the quality of offspring (duration of development from an egg to a new imago and time from emergence to death under starvation of new imagoes). The procedures for obtaining flea eggs and estimation of the offspring development and time to death under starvation are described elsewhere (e.g. Krasnov et al., 2001; Khokhlova et al., 2010a; Khokhlova et al., 2012; Khokhlova et al., 2014). In brief, fleas were randomly selected from the incubation-development boxes. An individual rodent was placed in a plastic cage (60×50 cm and 40 cm high) with a floor of 3–5 mm of clean sand covered by a wire mesh (5×5 mm). Then, 20 female and 10 male fleas were released into a cage and allowed to feed for 2 days. After 2 days of an uninterrupted stay in a rodent's cage, fleas were collected from both the cage substrate and rodent's body (over a white plastic pan with a toothbrush until no more fleas were recovered). We examined the fleas collected from the rodent's body and cage substrate under light microscopy (40× magnification) and counted the number of fleas (males and females separately), and selected those with blood in their midgut. Then, fleas recovered from the same rodent individual (those that took a blood meal) were placed in clean Petri dishes and then transferred to an incubator (FOC225E, Velp Scientifica srl) at 25°C air temperature and 75% relative humidity (RH) for 24 h. The next day, we checked the Petri dishes, collected fleas again and counted all eggs laid by all females in a given group. Then, we placed each individual rodent in a wire mesh (5×5 mm) tube (15 cm length and 5 cm diameter) that limited movement and did not allow self-grooming. Tubes containing rodents were placed in individual white plastic baths. Fleas previously collected from this same rodent were then released into the hair of the rodent. After feeding on a host for 6 h (the time necessary for acquiring a blood meal as estimated from our preliminary observations), fleas were collected and examined for blood in the midgut as described above. Fleas of each group with blood in their midgut were returned again to new Petri dishes and transferred to an incubator for 24 h, after which we again checked the Petri dishes and counted newly laid eggs. This procedure was repeated daily during the next 2 days. Based on the number of fed females and the number of eggs produced, we calculated for each group of fleas (that is, for each individual rodent) the mean number of eggs produced per fed female during 4 days of oviposition.

Petri dishes with eggs were checked daily till all the eggs hatched. Each larva hatched from the group of eggs was transferred into a new individual vial with clean sand and dry bovine blood (larvae nutrient medium). After a flea pupated, a vial was covered with a 5×5 cm nylon screen. Vials were checked once a day. Then, each imago was left in its vial under the same air temperature and RH and checked daily until its death. Vials with eggs, larvae, pupae/cocoons and new imagoes were maintained in the incubators (see above) at 25°C air temperature and 75% RH. The sex of each dead imago was identified by examination of its genitalia under a light microscope. For each new imago, we calculated total duration of development as the sum of the number of days from egg to larvae hatching, from larvae hatching to pupation and from pupation to emergence. We also calculated time to death under starvation by counting the number of days that each new imago stayed alive without access to a host.

Data analysis

For each individual rodent, we analysed LC, PHA response and the number of eggs produced per female flea during 4 days of oviposition when exploiting this rodent (dependent variables) using linear mixed-effects models (LME) (Zuur et al., 2009) with rodent sex and age at weaning as fixed explanatory variables. Because some rodents were born from the same mothers, the individual ID number of a mother was included as a random factor in each model, allowing us to control for individual variation among mothers. Duration of development and time to death under starvation (dependent variables) were analysed for each flea of the new generation using LME with rodent host sex and age at weaning and flea sex as fixed

explanatory variables. Because we fed a group of parent fleas on the same host individual, we needed to account for within-individual host non-independence of flea offspring. Individual variation among rodent mothers had to also be accounted for because some rodents were siblings. Consequently, the individual ID number of a host nested in the individual ID number of a rodent mother was included as a random factor in each of the two models. Inclusion of flea sex as a fixed variable in these models was necessary because the duration of development and resistance to starvation could differ between male and female fleas (Khokhlova et al., 2010b). We fitted the models using the lme function as implemented in 'nlme' package (version 3.1-108) (Pinheiro et al., 2013) in R (version 3.0) (R Development Core Team, 2013). After running the models, we used Tukey's HSD tests for multiple comparisons adjusted for mixed effects models to test for differences between rodents weaned at different ages in their effect on dependent variables using the glht function in 'multcomp' R package (version 1.3-2) (Hothorn et al., 2008). Reference levels for ordinal explanatory variables of rodent and flea sex were established arbitrarily as females. All dependent variables except PHA were log-transformed prior to analyses. Figures present untransformed data.

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