From endosymbionts to host communities: factors determining the reproductive success of arthropod vectors

Irit Messika, [1]

Mario Garrido, [2]

Hadar Kedem, [1]

Victor China, [3,4]

Yoni Gavish, [5]

Qunfeng Dong, [6]

Clay Fuqua, [7]

Keith Clay, [7]

Hadas Hawlena, [2]✉

Phone (+972) 08-6596775
Email hadashaw@bgu.ac.il

[1] Department of Life Sciences, Ben-Gurion University of the Negev, Beersheba, Israel

[2] Mitrani Department of Desert Ecology, Swiss Institute for Dryland Environmental and Energy Research, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, 84990 Midreshet Ben-Gurion, Israel

[3] Department of Zoology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

[4] The Inter-University Institute for Marine Sciences, Eilat, Israel

[5] Faculty of Biological Sciences, School of Biology, University of Leeds, Leeds, UK

6 Department of Public Health Sciences, Center for Biomedical Informatics, Stritch School of Medicine, Loyola University Chicago, Chicago, IL, USA

7 Department of Biology, Indiana University, Bloomington, IN, USA

Abstract

Elucidating the factors determining reproductive success has challenged scientists since Darwin, but the exact pathways that shape the evolution of life history traits by connecting extrinsic (e.g., landscape structure) and intrinsic (e.g., female's age and endosymbionts) factors and reproductive success have rarely been studied. Here we collected female fleas from wild rodents in plots differing in their densities and proportions of the most dominant rodent species. We then combined path analysis and model selection approaches to explore the network of effects, ranging from micro to macroscales, determining the reproductive success of these fleas. Our results suggest that female reproductive success is directly and positively associated with their infection by *Mycoplasma* bacteria and their own body mass, and with the rodent species size and total density. In addition, we found evidence for indirect effects of rodent sex and rodent community diversity on female reproductive success. These results highlight the importance of exploring interrelated factors across organization scales while studying the reproductive success of wild organisms, and they have implications for the control of vector-borne diseases.

AQ1

Keywords

Fitness
Life history
Model selection
Parasites
Path analysis
Scales

The online version of this article (doi:10.1007/s00442-017-3906-4) contains supplementary material, which is available to authorized users.

Introduction

The number and quality of offspring an individual produces, termed as reproductive success, affect the population dynamics, biotic interactions, and life history evolution of a species (Werner and Anholt 1993; Lindstrom 1999; Saccheri and Hanski 2006). However, revealing the factors determining an individual's reproductive success remains a major challenge in evolutionary ecology (Jorgensen et al. 2011; Rollinson and Hutchings 2013; Lim et al. 2014).

There is substantial variation in female reproductive success in nature (Clutton-Brock 1988). This variation has genetic and environmental components, which often interact (Ellegren and Sheldon 2008). Variation in reproductive success may also result from trade-offs that arise because females have a limited pool of resources. Accordingly, females may change their resource allocation to reproduction according to their intrinsic conditions and the expected intrinsic and extrinsic conditions of their offspring (e.g., Parker and Begon 1986).

At present, most studies have focused on either one or a few intrinsic or extrinsic factors at a time determining female reproductive success (e.g., Kudo 2001; Horn et al. 2005; Bashey 2006; Marshall et al. 2006; Khokhlova et al. 2014; but see Loot et al. 2011). Extrinsic factors include macroscale environmental conditions related to climate and landscape structure, and local conditions determined by the habitat type, presence of other organisms, and food availability (Clutton-Brock 1988; Major and Kendal 1996; Lindstrom 1999; Kudo 2001; Rosenfeld and Roberts 2004; Bashey 2006; Marshall et al. 2006; Baker et al. 2008). Intrinsic factors are related to the genetics and state of the reproductive female and include the presence of selfish genetic elements, group membership, age, and body condition (Clutton-Brock 1988; Fincke 1988; Carriere and Roff 1995; Rosenfeld and Roberts 2004; Burt and Trivers 2006; Donelson et al. 2008; Wilson et al. 2009). Adopting the broader view of the "holobiont" concept, which considers a multicellular organism and all its associated symbiotic microbes as one unit (Rosenberg and Zilber-Rosenberg 2011), introduces an additional class of intrinsic factors—the presence of associated microbial symbionts (Rosenfeld and Roberts 2004).

The symbionts can directly alter or indirectly affect host reproduction (Lefevre and Thomas 2008). However, in nature, extrinsic and intrinsic factors are often interrelated, and it is the exact pathways connecting these factors and the reproductive success of females that shape the evolution of life history traits.

Here we investigate the relative roles that extrinsic and intrinsic factors play, and how they interact in determining the current reproductive success of female fleas collected from wild rodents. Vectors, in general, and fleas, in particular, are convenient models in which to investigate these questions for several reasons. First, information on the pathways that connect the various extrinsic and intrinsic factors and the reproductive success of vectors is of particular interest for the control of vector-borne diseases. Second, the reproductive success of vectors is expected to be affected by both extrinsic and intrinsic factors because they play a role both as parasites of their vertebrate host, often actively choosing their host individuals (Krasnov et al. 2002b; Hawlena et al. 2007a), and as hosts themselves for their passenger microbes (Balashov 1984; Gillespie et al. 2004). Finally, fleas are able to adjust their allocation to offspring in accordance with their expected future environment (Khokhlova et al. 2014). Accordingly, we investigated how a suite of interrelated factors would correlate with the current reproductive success of female fleas by testing four non-mutually exclusive hypotheses ranging from micro to macroscales:

H1: Microscale: We expected to find neutral or positive associations between infection by vector-borne bacteria and the reproductive success of female fleas due to the dependency of these bacteria on the vector for transmission (Ewald 1983).

H2: Vector scale: We expected a positive association between the female fleas' reproductive success and their body size or mass and a negative association with their level of fluctuating asymmetry since reproductive success is likely to be higher for organisms with a better body condition (Honek 1993; Moller 1997). Moreover, the Trivers–Willard hypothesis (1973) and its later extensions predict that high-quality environments will enhance allocation towards the sex that has the greater reproductive value when the reproduction investment is high, e.g., female, in the case of fleas, whereas unfavorable conditions will favor allocation toward the "cheapest" sex (Sargent and Reid 1999; Seidelmann et al. 2010; Booksmythe et al. 2017). Thus, considering that a better body condition in female fleas reflects a

favorable environment, we also expected a positive association between the proportion of female offspring and female fleas' body size or mass and a negative association with their level of fluctuating asymmetry.

H3: Host scale: We expected that female fleas' reproductive success would be (1) greater on larger rodent species (*Gerbillus pyramidum*; Degen and Kam 1991) and the larger rodent sex (males; Krasnov et al. 2005c); (2) positively associated with rodent body condition, and (3) negatively associated with the flea and tick burden on the rodent (Hawlena et al. 2005, 2006a, b). This is because the reproductive success of parasites (vectors, in our case) is likely to increase with the quality of their host as a food resource (Tsai et al. 2001; Seppala et al. 2008; but see Krasnov et al. 2005b).

H4: Macroscale: We expected that female fleas' reproductive success would be negatively associated with rodent community diversity, since the latter reduces the likelihood of fleas to specialize on a given host species, but positively associated with total rodent density, which increases the likelihood of transmission of parasites (vectors, in our case) (Krasnov et al. 2002a; Lajeunesse and Forbes 2002). Moreover, the theory predicts that in heterogeneous environments, such as in areas characterized by high rodent community diversity, low synchrony in offspring emergence will benefit the parent by increasing the chances that at least some offspring will be preadapted to the environment and by decreasing the competition between siblings (Edgerly and Livdahl 1992; Olofsson et al. 2009). Accordingly, we also expected that synchrony in offspring emergence would be negatively associated with rodent community diversity.

Considering that female fleas might be energetically constrained, we also looked for correlative evidence for female trade-offs between (1) the number and mass of offspring; (2) the number and development time of the offspring, and (3) sex allocation (the investment in male versus female offspring; Fox and Czesak 2000; Roff 2002; Seidelmann et al. 2010).

We collected female fleas from wild rodents in plots differing in their densities and proportions of the most dominant rodent species and assessed their reproductive success. Our analyses suggest that a network of factors ranging from micro to macroscales determine the reproductive success of these fleas. These findings demonstrate the importance of simultaneously evaluating factors at different organizational scales for a better understanding of an individual's reproductive success and for evaluating the risk of vector-

borne diseases in natural communities.

Materials and methods

Study design

We trapped, tagged, measured and characterized rodents, sampled their fleas and ticks, and randomly collected one female flea from each host in plots differing in their proportions of the most dominant rodent species (*Gerbillus andersoni*, *G. pyramidum*, and *G. gerbillus*) and their total rodent density in the western Negev Desert in Israel (Table 1). Female fleas were allowed to lay eggs in separate vials, were measured for tibia length and leg asymmetry, and then were subjected to DNA extraction and PCR to screen for the most dominant bacteria (*Mycoplasma* and *Bartonella*; Gavish et al. 2014; Cohen et al. 2015b). These data allowed us to test the relative importance of the various factors (Table 2) in explaining the current reproductive success of fleas while searching for correlations that are indicative of reproductive trade-offs.

Table 1

The number of female fleas and their offspring number, with respect to host species and plot type (plots dominated by a single or multiple rodent species)

Plot type	Number of plots	Host species	Number of hosts trapped	Number of female fleas sampled	Range of litter size per female flea	Mean litter size per female flea
Single species	15	*G. andersoni*	56	37	0–6	1.4
		G. pyramidum	16	13	0–4	2.3
Multiple species	24	*G. andersoni*	51	41	0–5	1.7
		G. pyramidum	56	50	0–8	2.5
		G. gerbillus	15	7	0–2	0.6

Table 2

Model-weighted average regression coefficients with 95% prediction interval for the teste
synchrony (syn. in emergence), the mass variability (var. off. mass), the female flea mass

Ind./dep. (Hyp./cale)	Female reproductive success			Development time	
	Off. num	Res. off. num.	Sex ratio	M	F
Sample size	141	141	103	75	81
Mycoplasma	**0.23 ± 0.19**	**0.50 ± 0.45**	0.12 ± 0.70	0.02 ± 0.78	0.32
Bartonella	−0.16 ± 0.19	−0.30 ± 0.40	0.46 ± 0.64	NA	
Female size	**5.6 ± 4.1**	NA	−9.7 ± 15.6	11.4 ± 19.2	3.6
Female asymmetry	−3.9 ± 8.4	−9.0 ± 18.2	**33.8 ± 35.2**	NA	
Sp.	**0.37 ± 0.30**	**0.74 ± 0.63**	−0.01 ± 1.01	0.2 ± 1.1	0.14
Sex	0.04 ± 0.28	0.06 ± 0.58	−0.22 ± 0.94		
Body condition	0.011 ± 0.025	0.023 ± 0.058	0.015 ± 0.088	NA	
Flea burden	0.007 ± 0.011	0.015 ± 0.025	−0.015 ± 0.034		
Tick burden	−0.021 ± 0.04	−0.044 ± 0.089	−0.09 ± 0.14		
Host comm. diversity	0.01 ± 0.20	0.15 ± 0.42	**−0.84 ± 0.67**	−0.67 ± 0.78	−0.0
Host density	**0.069 ± 0.059**	**0.14 ± 0.12**	0.09 ± 0.20	−0.16 ± 0.23	0.15
Offspring numbers	NA			0.28 ± 0.35	0.23
Mean measured character	NA				

The model-set designed to explain the variability in offspring number was repeated af
male (M) and female (F) offspring. The tested factors are ordered from the microscale
et al. 2014) are marked in bold

N/A not applicable, *sp* species

Rodent trapping and ectoparasite collection

We trapped rodents in 39 independent 1-ha plots located in the western Negev

Desert in Israel (34°30′E and 30°55′N). Sampling areas were chosen based on species composition records from previous rodent studies with the goal of sampling the most common species assemblages in the area (see Gavish et al. 2014; Kedem et al. 2014, for details). The stability of the host-species composition in the plots over more than 3 years suggests that our data reflect long-term differences in the rodent-species composition rather than an ephemeral situation (Kedem et al. 2014). A single rodent species predominated in 15 plots, and two or more species predominated in 24 plots (Table 1).

To avoid confounding time and treatments, pairs of single-host species (single-species communities of either *G. andersoni* or *G. pyramidum*) and multiple-host plots (communities that included a combination of the species *G. andersoni*, *G. pyramidum* and *G. gerbillus*) within a given sampling area were sampled during the same trapping sessions. To increase independence, all plots were located at least 40 m apart from each other. The 40-m distance restricts the movement of individuals between plots as indicated by both a long-term capture–recapture study under the same settings and the current study. In particular, during the long-term study, only 19% of the rodents were recaptured in adjacent plots after 4 months (Cohen et al. 2015a), and in the current study, no movement of individuals between plots was detected. Trapping was conducted over the course of a month and a half (July–August), so that seasonal changes would not introduce differences in conditions between trapping nights.

During each trapping session, we captured rodents in either four or six 1-ha plots on three sequential nights using 40 live Sherman traps per plot (H. B. Sherman Traps, Tallahassee, FL, USA), baited with millet seeds, and uniformly placed (4 × 10 rows). For each plot, we determined the total host density (the minimal number of live individuals trapped over three nights; Krebs et al. 1969; H4) and the rodent community diversity (whether a plot was composed of a single or multiple species; H4).

All individual rodents were non-reproductive adults. Each captured individual was ear-tagged, identified to species and sex, and weighed, and the left hind foot length was measured for the assessment of its body condition (H3). The body condition (mass index) of the host was assessed according to Eq. 1, following Peig and Green (2009):

$$\text{Scaled mass index: } \hat{M}_i = M_i \left[\frac{L_0}{L_i} \right]^{b_{\text{SMA}}}, \qquad\qquad 1$$

where M_i and L_i are the body mass and hind foot length of host i, respectively; b_{SMA} is the scaling exponent estimated by the standardized major axis (SMA) regression of M on L; L_0 is the arithmetic mean value of L for the study population, and $\hat{M}_i$ is the predicted body mass for host i when the linear body measure is standardized to L_0.

To assess flea and tick burden (H3), we collected them from each rodent and counted them. Fleas and ticks were collected only from individuals in their first capture to avoid pseudo-replication. We gently held each rodent above a plastic can, blew air over its fur, and collected the fleas as they jumped off, following Hawlena et al. (2005). Ticks were often attached to the ears, mouth, or nose and were aggregated around open wounds. This aggregated pattern allowed us to count these parasites on the rodent body by carefully scanning the target areas before collecting a few specimens for further identification. The first female flea collected from each rodent individual was placed into a glass vial for evaluation of its current reproductive success and bacterial community composition (see below). All other fleas and ticks were counted and stored in 70% ethanol at −20 °C until their species was determined. All adult fleas were morphologically identified as *Synosternus cleopatrae*, and all larval and nymph ticks were morphologically identified as *Hyalomma impeltatum* (see also Hawlena et al. 2005). We released all rodents at their place of capture at the end of the procedure.

Assessment of female quality and current reproductive success

A single female flea was collected from each trapped rodent individual (Table 1). The sampled fleas were individually placed into a ventilated glass vial containing 3 mm of clean sterilized sand. We incubated the vials in a growth chamber (95% ± 3 relative humidity and 23 ± 2 °C) for 48 h. Then, we removed the female fleas and stored them in 70% ethanol at −20 °C until their body and legs were measured and their DNA extracted. Following the flea removal, we added to the vials, which contained sand, eggs, flea feces, and flea gut voids, a supplement of 150 µl of larvae grind and a filtered medium (94% dry bovine blood, 5% millet flour and local vegetation, and 1% ground rodent excrement). This supplement provided ad libitum food for the larva,

thus preventing intraspecific competition and increasing the chances that all larvae would survive, produce cocoons, and emerge as an imago (Krasnov et al. 2005a; Khokhlova et al. 2014). After 2 weeks of incubation, we monitored the vials every 2–3 days and collected newly emerged offspring into ethanol. We then determined the sex of the offspring and estimated their body size by measuring their maximum tibia length, a widely used comparative measure of body size in insects, which is fixed throughout the imago's life and is thus age-independent (e.g., Ellers et al. 2000; Tripet et al. 2002; Bezemer et al. 2005). Offspring body size was later converted to body mass (see below), which was used as a proxy for quality since, in insects, larger body mass within a species is associated with higher fecundity (Honek 1993). In fleas in particular, the ability of a new imago to survive long periods of starvation depends on the amount of energetic stores in its fat tissue, which accumulates during pre-imaginal development (Krasnov 2008).

To assess the quality of female fleas (H2), we estimated their body mass and fluctuating asymmetry, in which a heavier and more symmetrical female was considered as a higher quality female (Honek 1993; Palmer and Strobeck 2003). For this purpose, we measured, in triplicate, the right and left lengths of the coxa, femur and tibia of each female flea. The mean of the two tibia measurements (six measurements) was used as an approximation for the body size of each female flea. To convert the tibia lengths of both female and offspring to mass units, we raised the tibia measurements to the power of three. We chose this value since the power function of the relationship between length and body mass in terrestrial arthropods ranges from 1.0 to 6.7, but is rarely below 2 or above 4 (Schoener 1980; Ganihar 1997; Hódar 1997; Brady and Noske 2006; Martin et al. 2014). Regardless of this choice, the results of the statistical analysis were robust whether we used length or mass units. The level of fluctuating asymmetry reflects the ability of individuals to undergo stable development of their phenotype under a range of environmental conditions. Symmetrical individuals generally have faster growth, higher fecundity, and better survival rates than do more asymmetrical individuals (Moller 1997). The fluctuating asymmetry (FA) index was calculated after we found a strong indication for fluctuating asymmetry in the three organs (coxa, femur and tibia; data not shown), according to the FA17 index, following Eq. 2 (Palmer and Strobeck 2003):

$$\mathrm{FA17}_i = \frac{\sum \left| \ln \frac{R_j}{L_j} \right|}{T}, \qquad 2$$

where Rj and Lj are the means of the right and left triplicates for trait j, respectively; and T is the number of organs measured per individual (coxa, femur and tibia). Body and leg measurements were conducted with a stereomicroscope (DM500, Leica, Germany) equipped with a digital camera (UI-5580SE, IDS Imaging, Germany) and with the aid of the program ImageJ (National Institute of Mental Health, Maryland, USA).

We assessed the current reproductive success of female fleas with ten variables, including offspring number, sex ratio (female offspring proportion), mean development time from egg to imago, offspring mass, synchrony in offspring emergence, and variability in offspring mass (quantified by the standard deviation in tibia length) (Table 2).

Bacterial sampling

We extracted DNA from the sampled female fleas, using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) (Hawlena et al. 2013). Each set of extractions included a negative control (all the reagents except the flea).

We screened for *Mycoplasma* and *Bartonella* bacteria using PCR with the specific primers and reaction conditions detailed in Kedem et al. (2014; H1). The choice of these bacteria was based on previous evidence suggesting that both of them are common flea-borne bacteria in Negev Desert communities (Chomel et al. 1996; Woods et al. 2005, 2006; Morick et al. 2011, 2013a) and that they constitute the core bacterial communities in both rodent blood and fleas (Gavish et al. 2014; Gutiérrez et al. 2014; Cohen et al. 2015b). *Mycoplasma* bacteria in the Negev Desert belong to a single cluster, which is closely related to, but distinguishable from, *M. haemomuris* (Kedem et al. 2014). *Bartonella* bacteria belong to two or more clusters, but each cluster is found in both flea and blood samples from all three host species, and in plots with different host-species compositions (Gutiérrez et al. 2014; Kedem et al. 2014).

To foreclose the possibility that negative samples simply represented low-quality extractions, we added two additional assays, targeting the 18S gene of fleas and 16S general ribosomal bacterial DNA. Accordingly, we excluded from the following analyses six samples that were negative for all the PCR assays. Sanger sequencing was performed on 20% of the PCR-positive samples, using a PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) at the National Institute for Biotechnology in the Negev,

Beer-Sheva, Israel. Results confirmed that the tested bands indeed corresponded to sequences derived from *Mycoplasma haemomuris*-like bacterium and *Bartonella* sp.

Data analyses

Analyses were conducted in two stages. First, we searched for the most influential factors that best explained the variability in female quality and reproductive success (Table 2), and then, we quantified the causal pathways of the intrinsic and extrinsic factors, using a path analysis approach (Wootton 1994). For both stages, we compared models using model probabilities (w_i, where i corresponds to a specific model) based on an Akaike information criterion corrected for a small sample size (AICc), which gives a measure of the likelihood, on a 0–1 scale, that a particular model is the best model (Burnham and Anderson 2002). The combined path analysis-model selection approach evaluates alternative causal hypotheses regarding the directions of, the links between, and the strength of interactions (e.g., Cohen et al. 2015a). In addition to the insights gained from traditional regression-based methods, this approach can incorporate multiple interrelated dependent variables (e.g., female reproductive success and body mass), and can distinguish between direct and indirect effects (e.g., the effect of community diversity; Fig. 1).

Fig. 1

Path analysis model predicting the reproductive success of female *Synosternus cleopatrae* fleas. The *solid arrows* represent direct (a *single arrow* linking two variables) and indirect (*multiple arrows* passing through a mediator variable) influences included in the best model (model 1 in Table 2, $w_i = 24\%$). The *dashed arrows* represent additional relationships that are included only in the third and fourth best models (described in Table S1). Numbers on the *arrows* are standardized path coefficients, representing the relative strength of the given effect (β/SE). Plots *a–j* each illustrate the relationship described in writing and indicated by an *arrow* between the explanatory factor and the dependent variable in the path analysis wherein the main title is related to the *Y* axis. Plots are provided only for the most influential ($|\beta/\text{SE}| > 1$) direct effects while *error bars* represent standard errors. dens. = density; GA = *G. andersoni*; GP = *G. pyramidum*; myc. = *Mycoplasma*; infec. = infection; homogeny. = homogeneity; heterogen. = heterogeneity; asym. = asymmetry
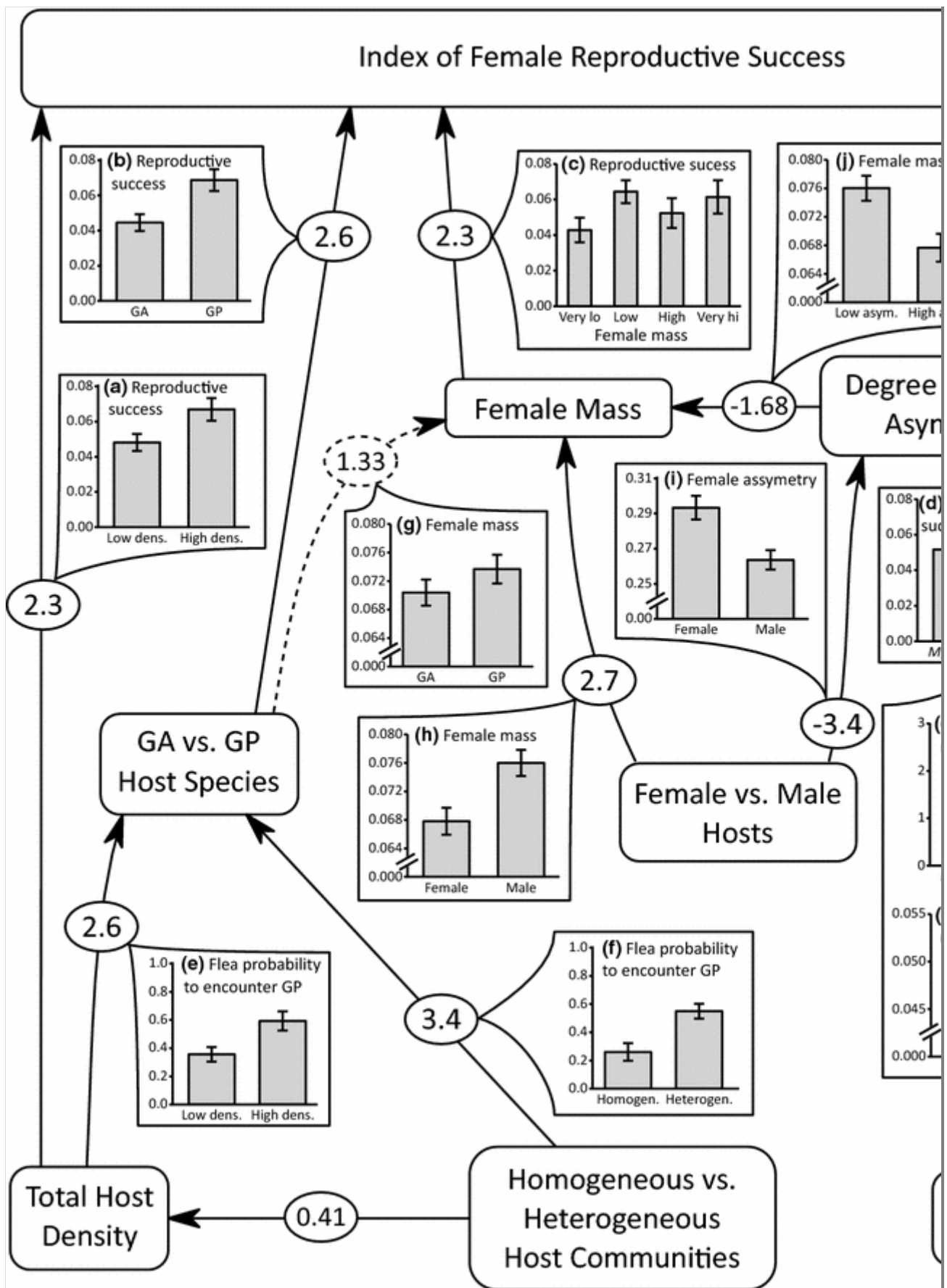
In stage 1, to select the most influential factors that best explained the variability in female quality and reproductive success, we explored 13 model-sets, each with a different dependent variable. In 10 of the model-sets, the

dependent variable was related to the current reproductive success of female fleas, while in three of the model-sets, it was related to the female quality (Table 2). The explanatory factors were related to all four hypotheses (H1–H4) and ranged from microscale factors (female infection by *Mycoplasma* or *Bartonella*) through vector-related factors (female mass and asymmetry) and host-related factors (host species, sex, and body condition, flea or tick burden on a host) to macroscale factors (host community diversity and host density). Apart from the high correlation between "host species" and "flea burden" ($r = 0.6$), there were only weak correlations between the explanatory factors ($|r| < 0.3$ for all other paired correlations).

To ensure an unbiased ranking of influential factors, each model-set included all possible combinations of additive nested Generalized Linear Models (GLM). For example, if a model-set included three factors (F1, F2 and F3), we fitted a total of eight GLMs including: a null model, and models including F1, F2, F3, F1+F2, F1+F3, F2+F3, or F1+F2+F3. This procedure ensures that all factors are equally represented in each of the model-sets. Following Johnson and Omland (2004), we scored each model using AICc and calculated their AICc weight (wAICc). Then, we assessed the direction of explanatory factors based on the averaged regression coefficients, using wAICc as weights. We listed a factor as important in a given model-set if its relative importance score (the sum of the AICc weights of all the models that included the focal factor) was larger than 0.7 (Wagner et al. 2014). In all analyses at this stage we used the function '*glm*' in the 'AICcmodavg' package in R (R-Core-Team 2013). Additional details are provided in the Electronic Supplementary Material (ESM).

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Stage 2 began with the construction of a saturated path model (Pedhazur 1983), which explored the effects of all the important factors revealed in stage 1 (relative importance >0.7; Wagner et al. 2014; Fig. S1) on an integrated index of female reproductive success (RS). The index was calculated following Eq. 3 (details in the ESM):

$$\text{RS} = \sum_{i}^{\text{NF}} \text{BM}_\text{F} + \sum_{i}^{\text{NM}} \text{BM}_\text{F}, \qquad 3$$

where NF and NM are the total number of female and male offspring in a clutch, respectively, and BM_F and BM_M are the body masses of female and

male offspring *i*, respectively. We then compared different model versions modified from the saturated models while using the same mediators and dependent variables (ESM, Table S1). The path analysis was conducted with the Mplus software [(Muthén and Muthén 2012); Los Angeles, CA, USA]. Additional details on the statistical analyses are provided in ESM.

Results

Overall, 148 female fleas were collected from 148 hosts and 39 plots (Table 1); their DNA was of high quality (see above), and they produced 279 flea offspring. The number of offspring per female ranged from 0 to 8 (mean ± SE; 1.89 ± 0.14), with most female fleas producing from 0 to 2 offspring. The overall sex ratio of the 279 offspring was not significantly different from 1:1 (130 males and 149 females; $G = 0.402$, $df = 2$, $p = 0.82$). The female offspring developed significantly faster than the male offspring with medians of 33 and 41 developmental days, respectively (25–75% quartile range: 32–34 and 41–43, respectively). The emergence of siblings of the same sex was mostly synchronized. The longest range of sibling emergence was 8 days for female siblings and 15 days for male siblings. Female offspring had longer tibias than males (mean ± SE: 0.46 ± 0.002 and 0.37 ± 0.001, respectively; $t = -31$, $df = 138$, $p < 0.001$). Of the female fleas, 42% were infected by *Bartonella* and 22% by *Mycoplasma*. Since only seven females were collected from *G. gerbillus* hosts, we removed them from all of the following analyses.

The best factors predicting offspring number were infection by *Mycoplasma* (H1), female mass (H2), host species, (H3), and total host density (H4) (Table 2). These results were robust even when we repeated the analysis using the residuals of "the offspring number on female mass" as a dependent variable ("res. off. num."; Table 2). The best factors explaining the offspring sex ratio were female asymmetry (H2) and community diversity (H4). The best factors explaining the male and female offspring mass were infection by *Mycoplasma* (H1) and community diversity (H4), respectively. Other than the effect of offspring number on emergence synchronization, none of the tested factors sufficiently predicted variability in the development time and emergence synchronization of the offspring, or variability in their mass (Table 2). The effect of offspring number is most likely the result of a statistical artifact given that the chance of offspring emerging on separate days increases with offspring number. The best factors predicting both variability in female mass and asymmetry level was host sex, whereas none of

the tested factors successfully explained variability in infection by *Mycoplasma* (Table 2). The only indication for a female trade-off was between the number and mass of the offspring, in which infection of female fleas by *Mycoplasma* was associated with a greater offspring number but a lower mean body mass of male offspring (Table 2; Fig. 1d).

Four out of 42 candidate path analysis models had good support from the data ($w_i > 10\%$; $\sum w_i = 66\%$; Fig. 1 and Table S1 in ESM). In all four, the index of female reproductive success was directly and positively associated with total host density and female mass, and was higher for females collected from *G. pyramidum* than for those collected from *G. andersoni* (Fig. 1a–c). In three of the four models, there were also positive associations between *Mycoplasma* presence in the female and its index of reproductive success (Fig. 1d). Another direct, but negative, association was revealed between female asymmetry and its index of reproductive success. However, the effect size of this association was weak (< -1), and it was included in only one of the four models (ESM, Table S1). The four best path analysis models all indicate that the effect of community diversity on female reproductive success is not direct, but rather mediated by changes in host densities and in the female's probability of encountering each of the host species (Fig. 1e–f). The models also indicate indirect effects of host species and sex on female reproductive success, mediated by changes in female mass and asymmetry (Fig. 1g–j).

Discussion

From micro to macrofactors determining female reproductive success

Our overall hypothesis that a suite of interrelated factors, ranging from micro to macroscales, is associated with the current reproductive success of female fleas was confirmed. Agreeing with our hypotheses, we found positive associations between the index of female reproductive success and (1) its infection by *Mycoplasma* bacteria (H1, microscale); (2) its body mass (H2, vector scale); (3) the body size of the host species (H3, host scale), and (4) the total host density (H4, macroscale). These results, together with evidence from previous studies (e.g., Bezemer et al. 2005; Horn et al. 2005; Bashey 2006; Baker et al. 2008), offer explanations for the observed variation in reproductive success of wild organisms and suggest that a major component for this variation is environmental. In particular, the environmental component in these systems is largely determined by the quality of the habitat (or host),

both at the fine (H3, host scale; Seppala et al. 2008; Loot et al. 2011) and coarse (H4, macroscale; Horn et al. 2005; Segoli and Rosenheim 2013) scales, and by biotic interactions (H1, microscale; Kudo 2001). The path analysis further suggests that the factors constituting this suite are interrelated (Fig. 1), and thus that the focus on a sole explanatory factor may be misleading. For example, the occasional failure to find an association between the organism's body size or mass and reproductive success (Tepedino and Torchio 1982; Woog 2002; Dickerson et al. 2005; Bosch and Vicens 2006; Herreras et al. 2007) may be due to the presence of endosymbionts, which may simultaneously influence both traits. It remains to be tested whether the observed associations are the result of plastic strategies in response to the various factors or the direct result of selective pressures exerted on the females.

Why were the four factors above directly associated with the reproductive success of females, while the other factors, which were measured at the same scales, were not? It is likely that the factors tested differ in the selection pressures they impose on females. The theory predicts that organisms will invest more in overcoming factors that more frequently emerge as major constraints to fitness (Rosenheim et al. 2010; Segoli and Rosenheim 2013). The lack of a direct link between female reproductive success and infection by *Bartonella* (H1), asymmetry (H2), rodent sex (H3), and community diversity (H4) supports this theory. First, *Bartonella* has negligible effects on the feeding and reproductive parameters of fleas (Morick et al. 2013b). Second, female asymmetry is expected to constitute a weaker constraint on reproductive success than body mass, which can limit the number of eggs in a clutch (Krasnov 2008). Third, it is likely that males and females from the same host species constitute a more similar habitat for parasites (or vectors) than different host species of the same sex. Finally, host community diversity may be less relevant to parasites (or vectors) than the total host density since, in most cases, the parasite transmission rate is lower between species than within a host species (Begon et al. 1999).

Nevertheless, differences in selection pressures cannot fully explain the lack of associations between female reproductive success and either rodent body condition or flea burden (H3, host scale), since both factors are known to affect the fitness-correlated traits of fleas (Ma 2000; Krasnov et al. 2005b; Hawlena et al. 2007b; Tschirren et al. 2007). Alternatively, since both rodent body condition and flea burden fluctuate over time (Khokhlova et al. 1994,

2001; Krasnov et al. 2006), they may not constitute reliable cues for female reproductive strategies (Fischer et al. 2011).

Weak evidence for female reproductive trade-offs

Various theoretical models have been developed to explain the substantial variation observed in offspring number and quality among species and individuals, and even among offspring within a clutch. Most assume a trade-off between offspring number and size or mass (e.g., Roff 1992; Stearns 1992). The only support for a reproductive trade-off in our system comes from the finding that *Mycoplasma*-infected females had a higher number of offspring with a lower mean male offspring mass than did *Mycoplasma*-free females (Fig. 1d). The conflicting effects of *Mycoplasma* on the number and mass of offspring may represent a trade-off that is mediated by differential female allocation of resources to egg maturation and development. It is still unknown whether *Mycoplasma*-free females are in poorer nutritional states than *Mycoplasma*-infected females. However, if they are, the observed number–mass relationships are consistent with the general prediction that larger but fewer offspring are expected in lower quality environments than in better quality environments (Parker and Begon 1986; Bashey 2006; Rollinson and Hutchings 2013). An indication for a number–quality trade-off was also found in *Xenopsylla ramesis* and *Parapulex chephrenis* fleas, which laid more eggs but had lower quality offspring when feeding on their principal host than those feeding on more phylogenetically distant hosts. In these cases, too, the substantial differences between the host species could constitute a clear indication of an offspring number–mass trade-off (Khokhlova et al. 2013, 2014).

Nevertheless, we cannot rule out the possibility that *Mycoplasma*, like other symbiotic bacteria (Lefevre and Thomas 2008), manipulate female fleas to produce more offspring with the goal of increasing the bacterial transmission probability and reproductive success. To distinguish between the "flea trade-off" and the "*Mycoplasma* manipulation" alternative explanations, future studies should compare the number of flea offspring of *Mycoplasma*-infected and *Mycoplasma*-free females under a gradient of nutrient regimes supplied to the fleas.

Our failure to detect a direct relationship between offspring number and mass, as well as other life history trade-offs, may be due to the correlative nature of our study in which substantial natural variation in reproductive efforts

obscures potential trade-offs. For example, in natural populations, young females in good condition, who can produce high numbers of offspring with high energy invested per offspring, coexist with females who can only afford low-quality offspring in low numbers (Reznick et al. 2000; Lim et al. 2014). Further experiments should therefore explore the trade-off between offspring number and mass in fleas and other vectors.

Significance of a holistic approach

Here we took a holistic approach to studying the current reproductive success of organisms living in complex natural communities. By combining path analysis and model selection approaches, we highlighted the most likely pathways that may connect a suite of interrelated factors, ranging from female flea endosymbionts to the community structure of the rodent hosts, and to the reproductive success of fleas. The conceptual network of connections offered by our best models produced specific hypotheses that should be experimentally verified, including potential mechanisms that would not have been revealed by classical regression methods. For example, the results of the first stage analysis supported direct associations between host community diversity and female offspring mass. This could have been used as support for the conservative bet-hedging theory, which predicts that offspring quality would increase with environmental diversity (Olofsson et al. 2009). However, the second stage analysis suggests that the effect of host community diversity on the reproductive success of females is indirect, mediated by changes in total host density and species composition (Fig. 1).

In the specific case of vectors, a holistic approach to the study of reproductive success should be crucial when making management decisions. For example, our results suggest that risk assessment for vector-borne diseases should be conducted separately for communities of hosts varying in their species composition and for different host species. Moreover, the results of our case study support previous recommendations to consider the body mass of vectors and the potential impact of their endosymbionts during the design of biological control actions (e.g., release of sterile or endosymbiotic-infected vectors; Zindel et al. 2011; Yuval et al. 2013; Segoli et al. 2014; Calvitti et al. 2015). Finally, a mechanistic approach, which differentiates between direct and indirect effects, is important for understanding observed field correlations. To this end, our results provide insights into the mechanisms that determine the indirect effects of community diversity on disease risk (e.g., Johnson et al. 2013; Salkeld et al. 2013; Wood et al. 2014). By using a

mechanistic approach, we can also exploit possible constraints on the evolution and epidemiology of vector-borne diseases. For example, in our system, it is likely that clearing fleas of *Mycoplasma* would result in flea population suppression (see a theoretical example in Sisterson 2009).

Taken together, our study demonstrates that a holistic approach may generate general applied and basic science insights into the mechanisms of variation in the reproductive success of wild organisms. However, since species may vary in their constraints to fitness and in the reliability of their cues for female reproductive strategies, the specific factors determining reproductive success may be system-specific.

Acknowledgements

Data accessibility   Raw data and statistical scripts can be accessed via the public archive "Figshare.com". Accession addresses are http://dx.doi.org/10.6084/m9.figshare.4818121 and http://dx.doi.org/10.6084/m9.figshare.4818136, respectively.

Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary material 1 (XLSX 82 kb)

Supplementary material 2 (DOCX 53 kb)

Supplementary material 3 (XLSX 27 kb)

Supplementary material 4 (DOCX 23 kb)

Supplementary material 5 (DOCX 90 kb)

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