Evol Ecol (2016) 30:1081–1095
DOI 10.1007/s10682-016-9861-4

ORIGINAL PAPER

Variation of clutch size and trophic egg proportion in a ladybird with and without male-killing bacterial infection

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Received: 19 April 2016 / Accepted: 9 September 2016 / Published online: 13 September 2016

Abstract Maternally inherited bacterial endosymbionts can kill male embryos of their arthropod hosts to enhance the transmission efficiency of the endosymbionts. The resources from killed male eggs can be reallocated to infected female hatchlings as additional maternal investment. As a result, the number of offspring per patch and the maternal investment per offspring are expected to differ from the original optimal values for the host mother. Thus, in response to infection, these trait values should be adjusted to maximize the lifetime reproductive success of host females and the fitness of inherited endosymbionts as well. Here, we examined clutch size, egg size, and the proportion of trophic eggs (i.e., production of unhatched eggs, a maternal phenotype) per clutch of host mothers infected with male-killing bacteria. First, we developed a mathematical model to predict the optimal clutch size and trophic egg proportion in uninfected and infected females. Next, we experimentally compared these life-history traits in a ladybird, *Harmonia yedoensis*, between females infected or uninfected with male-killing *Spiroplasma* bacteria. Consistent with our predictions, clutch size was larger, egg size was smaller, and trophic egg proportion was lower in infected *H. yedoensis* females, compared with uninfected

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Electronic supplementary material The online version of this article (doi:10.1007/s10682-016-9861-4) contains supplementary material, which is available to authorized users.

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females. To our knowledge, this is the first empirical demonstration of variation in these life-history traits depending on infection with bacterial endosymbionts.

Keywords Bacterial endosymbionts · *Harmonia* · Male-killer · Maternal investment · *Spiroplasma*

Introduction

Many species of arthropods are infected with intracellular bacteria; in fact, one meta-analysis has estimated that more than 60 % of insect species harbour *Wolbachia*, the most-examined genus of facultative intracellular bacteria in arthropods (Hilgenboecker et al. 2008). Because intracellular bacteria, similar to mitochondria and chloroplasts, are transmitted from mothers to offspring via the egg's cytoplasm, the female sex is more valuable for the evolutionary success of such bacteria. This situation leads the bacteria to manipulate the reproduction of their host in diverse ways, including by inducing parthenogenesis, feminization of genetic males, and cytoplasmic incompatibility, and through male-killing behaviour (Stouthamer et al. 1999; Bandi et al. 2001; Weeks et al. 2002; Werren et al. 2008; Engelstädter and Hurst 2009).

Bacteria that use the male-killing strategy selectively kill their host's male embryos at an early developmental stage (Hurst and Majerus 1993; Hurst et al. 1997; Hurst and Jiggins 2000; Majerus and Majerus 2012; Fig. 1). Because this strategy decreases the number of hatchlings in an oviposition patch by approximately a half, competition among the infected female hatchlings for resources is reduced (Jaenike et al. 2003). Moreover, the dead male eggs constitute an additional maternal resource on which the infected female hatchlings can feed before they begin to forage on their own (Hurst and Majerus 1993). These changes

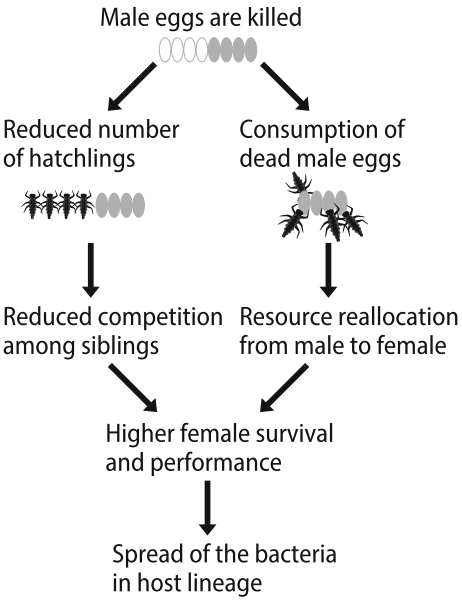

Fig. 1 This diagram depicts the strategy of male-killing bacteria (intracellular bacteria that kill the male eggs of their host). Male eggs in an infected clutch (*grey ellipses*) do not hatch. As a result, resource competition among their siblings, and other negative density-dependent factors, is reduced. In some host species, female hatchlings eat the killed male eggs, which thus constitute an additional maternal investment. This consumption may enhance hatchling performance with respect to, for example, prey capture efficiency and starvation tolerance. These benefits together enhance growth and survival rates of host females, which ultimately leads to an increased prevalence of male-killing bacteria

confer a fitness advantage on the host females and enhance the spread of male-killing bacteria in the host population (Hurst 1991; Elnagdy et al. 2011; Fig. 1), although failure to produce the rare sex (i.e., male offspring) may entail reduction in reproductive success in the female-biased population.

Recently, studies have shown that infection with intracellular bacteria can modify the host phenotype in ways that do not directly affect reproduction (reviewed in Feldhaar 2011; Ferrari and Vavre 2011; Goodacre and Martin 2012; Miller and Schneider 2012). The modified traits include physiological traits such as thermotolerance (Brumin et al. 2011) and developmental time (Elnagdy et al. 2013), nutrition provisioning (Brownlie et al. 2009), resistance against pathogens and parasites (Hedges et al. 2008; Bian et al. 2010; Jaenike et al. 2010), adult dispersal (Goodacre et al. 2009) and body size (Elnagdy et al. 2013), and oviposition site selection (Vala et al. 2004; Kenyon and Hunter 2007). When the bacteria manipulate host reproduction, they are expected to also modify other phenotypic characteristics of their host so as to enhance their transmission rate throughout subsequent host generations. From the viewpoint of the host mothers, these phenotypic adjustments due to infection with the reproductive manipulator improve their lifetime fitness. Therefore, modifications to various phenotypic characteristics of the host, in addition to the reproductive manipulation, are in the interest of both the bacteria and the host mothers.

Obviously, the number of hatchlings in a clutch is a life-history trait that is inevitably influenced by the presence of a male-killer infection. When male-killing bacteria invade an intact host population in which females produce optimally sized clutches, the number of hatchlings per clutch should decrease by approximately a half. This decrease means that the number of mature offspring from the oviposition patch will also decrease if the infected daughters do not acquire a fitness advantage that compensates for the death of the sons. Therefore, we hypothesized that clutch size will increase to restore the optimal number of hatchlings in the patch (Fig. 2a). Male-killing infection also affects the amount of maternal investment per offspring. In some animals, such as some species of land snails, bugs, and ladybirds, a part of the eggs in a clutch do not hatch, even in the absence of manipulation by male-killer bacteria; these eggs are then consumed by those offspring that do hatch (Crespi 1992; Perry and Roitberg 2006). These unhatched eggs that are consumed by the hatchlings (hereafter called "trophic eggs") are often considered to constitute an additional maternal investment in the hatched offspring and their production to be an evolved maternal phenotype, rather than unavoidable constraints (Crespi 1992; Perry and Roitberg 2006). Indeed, empirical findings have shown that mothers that provide trophic eggs enhance the starvation tolerance and feeding capacity of their offspring (Osawa 1992; Kudo and Nakahira 2004, 2005; Baba et al. 2011). When both the trophic eggs and the eggs with killed male embryos are reallocated to the hatched daughters, the amount of maternal investment per daughter can be expected to exceed the optimal investment amount of the mother. Therefore, we hypothesized that the proportion of trophic eggs in a clutch will decrease in infected mothers (Fig. 2b). Furthermore, infected mothers might produce smaller egg size to avoid excess of maternal investment per hatchling.

However, there was a controversy over the theoretical predictions of clutch size evolution in populations with male-killing bacteria. Hurst and McVean (1998) demonstrated that infected mothers increase their clutch size to forestall a large reduction of the hatchling number (Fig. 2a). On the other hand, the peculiar population genetics of male-killer-infected populations might hamper evolution of larger clutch size in infected individuals. Since infected females have to mate with uninfected males every generation, nuclear genes within the infected individuals are diluted with nuclear genes of uninfected individuals

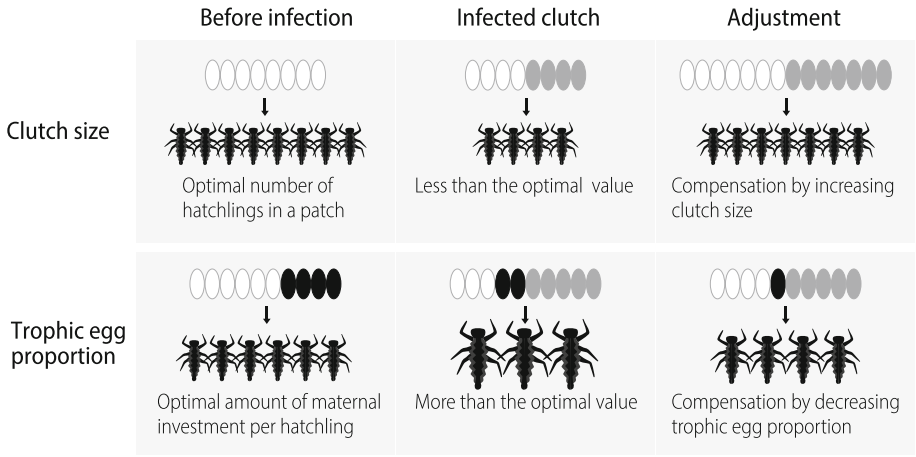

Fig. 2 Schematic diagram of the predicted responses with respect to life-history traits of a ladybird infected with male-killing bacteria. *White*, *grey*, and *black ellipses* represent viable eggs, male eggs killed by the bacteria, and trophic eggs, respectively. *Top row* Clutch size that optimizes the number of offspring in a patch. When an uninfected population with the optimal clutch size (*left column*) becomes infected with male-killing bacteria, the number of hatchlings becomes less than the optimal value (*middle column*). In this case, the infected mother should produce a larger clutch size to compensate for the reduction in the number of viable eggs (*right column*). *Bottom row* Proportion of trophic eggs that optimizes the amount of maternal investment per offspring. When a hitherto uninfected population with the optimal proportion of trophic eggs (*left column*) becomes infected with male-killing bacteria, the amount of maternal investment per offspring may exceed the optimal value for the mother, because both dead male eggs and trophic eggs are allocated to the hatched daughters (*middle column*). In this case, the infected mothers should reduce the number of trophic eggs provided (*right column*). The size of the ladybird larva in the *bottom row* roughly indicates the amount of maternal investment per hatchling

(Jaenike 2007). This leads to the situation that newly arisen beneficial mutations in infected individuals will quickly disappear from the population (Engelstädter and Hurst 2007). These population genetic mechanisms seem to reject the hypothesis of Hurst and McVean (1998). Nevertheless, variation in clutch size and other life-history traits could be maintained even in uninfected individuals of the population due to environmental uncertainty and some genetic constraints. This makes it possible that beneficial alleles for infected individuals are maintained even in uninfected males, and its frequency increases in infected females especially when selective advantage is high. Moreover, gene flow from infected females to uninfected males occurs when vertical transmission rate of bacteria is imperfect (Jaenike 2007). Furthermore, even though host species cannot adjust their phenotype, male-killing bacteria might manipulate host phenotypes to enhance their transmission rate. Therefore, a simple mathematical model should be still useful to predict optimal behavior and it is valuable to examine whether the predicted responses can be indeed observed in male-killer-infected populations.

In this study, we compared clutch size, trophic egg proportion, and egg size between females infected with male-killing bacteria and uninfected females. Our specific hypotheses are clutch size is larger, trophic egg proportion is smaller, and egg size is smaller in infected than in uninfected females. We firstly developed a simple mathematical model that is based on animal behaviour to predict the optimal clutch size and trophic egg proportion. This model considers consumption of trophic eggs and/or dead male eggs contributes to increase feeding rate and to the survival rate of hatched daughters once they

are foraging for food. Next, we experimentally examined the life-history traits in a predatory ladybird, *Harmonia yedoensis* Takizawa, between females infected with male-killing *Spiroplasma* (Noriyuki et al. 2014) and uninfected females. Despite the previous theoretical work (Hurst and McVean 1998), the predicted variation in clutch size and other life-history traits have not been detected in ladybirds infected with male-killing bacteria (Majerus 2003). Our approach incorporating mathematical model can analyse environmental conditions that favour the evolution of life-history traits especially in *H. yedoensis*, which specializes on the highly elusive prey and requires the large amount of maternal investment after hatching (Noriyuki et al. 2011).

Materials and methods

Model

We assume an insect species that lays some egg batches in a homogeneous habitat and hatched offspring (larvae) that eat a local food resource or capture prey for their development. Because we want to explain variation in maternal decision-making as a function of male-killer infection, we must define the optimal behaviour of an uninfected individual for reference. Thus, we first develop a basic mathematical model for optimal clutch size. Next, we add consideration of the proportion of trophic eggs in a clutch to the basic model. Then, we analyse the effects of male-killer infection on the optimal values of clutch size and trophic egg proportion. In modelling the trophic egg proportion, we consider two cases: Case 1, in which it affects feeding ability (e.g., catch rate of prey), and Case 2, in which the trophic egg proportion affects starvation tolerance. We also develop a simple model that incorporated sibling cannibalism (i.e., consumption of viable sibling's eggs rather than trophic eggs) to examine whether sibling cannibalism can evolve after the optimization of clutch size and trophic egg proportion (see Parker et al. 2002; Perry and Roitberg 2005a).

Clutch size

Clutch size often exhibits negative density dependence in a patch (Parker and Courtney 1984; Parker and Begon 1986). Moreover, the optimal clutch size is often affected by the cost of travelling between patches that a mother oviposits. Here, the negative density dependence of clutch size is expressed as the survival rate, which depends on the amount of food resources available for each offspring, and travelling cost is expressed as the travel time between patches. Let clutch size be E, and the amount of food per larva be x; x is affected by the feeding rate of the offspring on the food resource (V), food density (N) as well as by E, as follows: $x = VN/E$. We express the survival rate of the larvae, which is a function of the amount of food $[S(x)]$, by a saturation curve as follows: $S(x) = s_1x/(1 + s_2x)$ (see Table 1 for explanation of parameters). Since $x = VN/E$, this equation can be rewritten as $S(E) = s_1VN/(E + s_2VN)$. The cost of travelling depends on the travel time between patches (T_m) and the oviposition time per egg (T_e). A long travel time between patches reduces the number of oviposition patches in which eggs are laid. If the number of oviposition patches is m and total time for oviposition is T, then $m = T/(T_m + T_eE)$.

Maternal fitness is the product of the number of oviposition patches (m), clutch size (E), and the survival rate of the larvae $[S(E)]$. Thus, maternal fitness $W(E)$ is given by

Table 1 Parameters included in our model

Symbols	Definition	Condition
E	Clutch size	$E > 0$
p	Trophic egg proportion	$0 \leq P < 1$
x	Amount of food per larva	$x > 0$
y	Amount of trophic egg consumption per larva	$y \geq 0$
N	Food density	$N > 0$
T_m	Travel time between oviposition patches	$T_m > 0$
T_e	Oviposition time per egg	$T_e > 0$
T	Total time for oviposition	$T = m(T_m + T_eE)$
m	Number of oviposition patches	$m = T/(T_m + T_eE)$
s_1	Parameter of the survival rate function (s_1/s_2 is the maximum survival rate)	$s_1 > 0$, $0 < s_1/s_2 \leq 1$
s_2	Parameter of the survival rate function ($1/s_2$ is the point at which the survival rate is half the maximum rate)	$s_2 > 0$
V	Feeding rate	$0 < V < 1$
v_1	Feeding rate when offspring eat no eggs in Case 1	$0 < v_1 < v_2/v_3$
v_2	Parameter of the feeding rate function in Case 1 (v_2/v_3 is the maximum catch rate)	$v_2 > 0$, $v_1 < v_2/v_3 \leq 1$
v_3	Parameter of the feeding rate function in Case 1	$v_3 > 0$
s_3	Survival rate when offspring eat no eggs in Case 2	$0 < s_3 < s_4/s_5$
s_4	Parameter of the survival rate function in Case 2 (s_4/s_5 is the maximum survival rate)	$s_4 > 0$, $s_3 < s_4/s_5 \leq 1$
s_5	Parameter of the survival rate function in Case 2	$s_5 > 0$

$$W(E) = m \times E \times S(E) = \frac{T}{T_m + T_eE} \times E \times \frac{s_1VN}{E + s_2VN} \quad (1)$$

Optimal clutch size (E^*), that is, the clutch size that maximizes fitness for the mother [Eq. (1)] in the absence of infection, is expressed as $E^* = \sqrt{s_2VNT_m/T_e}$. The optimal clutch size with male-killer infection is represented as $E^* = \sqrt{2s_2VNT_m/T_e}$. Please see Appendix S1 for these calculations.

Trophic egg proportion

The proportion of trophic eggs in a clutch affects the survival rate of the larvae by decreasing the number of hatchlings and increasing their feeding performance or starvation tolerance. Here, we consider the case where the trophic egg proportion affects only feeding rate, not starvation tolerance before local food consumption (Case 1). The feeding rate increases as the number of trophic eggs consumed per larva increases (cf. Noriyuki et al. 2011). If the proportion of trophic eggs produced relative to total eggs laid is p, then the number of trophic eggs consumed by each larva (y) is given by $p/(1-p)$. The feeding rate, which is a function of the number of trophic egg consumed [$V(y)$], is expressed by a saturation curve that is represented as $V(y) = (v_1 + v_2y)/(1 + v_3y)$. $V(y)$ has intercept v_1 because larvae can catch prey even if they do not eat trophic eggs (see also Fig. S1). The

amount of food per larva (x) and the survival rate of larvae [$S(x)$] become $x = V(y)N/[E(1-p)]$ and $S(x) = s_1V(y)N/[E(1-p) + s_2V(y)N]$, respectively. Maternal fitness as a function of the proportion of trophic eggs in Case 1 [$W_{T1}(E,p)$] becomes

$$W_{T1}(E,p) = \frac{T}{T_m + T_eE} \times E(1-p) \times \frac{s_1[v_1(1-p) + v_2p]N}{E(1-p)(1-p+v_3p) + s_2[v_1(1-p) + v_2p]N} \quad (2)$$

Please see Appendix S2 for the calculation of optimal clutch size E^*_{T1} and optimal trophic egg proportion p^*_{T1} in Case 1. In our analysis, the optimal trophic egg proportion can sometimes become negative. In those instances, we set p^*_{T1} to zero, and E^*_{T1} is $E_{T1}(0)$.

In Appendix S3, we also consider the case in which the trophic egg proportion affects starvation tolerance before local resource consumption or prey capture occurs (Case 2).

Effect of male-killer infection on clutch size and trophic egg proportion

Next, we consider the effect of male-killer infection on the optimal clutch size and trophic egg proportion. We assume that hatched offspring do not discriminate between trophic eggs and eggs that do not hatch because of the male-killing bacteria infection, because no such discrimination skill has been reported in empirical tests. Given perfect vertical transmission of male-killing bacteria (i.e. no male eggs hatch) and sex-ratio parity, then the number of hatchlings in an infected clutch is given by $0.5(1-p)E$. The total number of trophic eggs and killed eggs consumed per larva is given by $(1 + p)/(1-p)$.

Optimal clutch size $\left(E^*_{T,K}\right)$ and optimal trophic egg proportion $\left(p^*_{T,K}\right)$ in the presence of male-killer infection is simply calculated as,

$$\left(E^*_{T,K}, p^*_{T,K}\right) = \begin{cases} \left(E_{T,K}(0), 0\right) : p^*_T \leq 0.5 \\ \left(E^*_T, 2p^*_T - 1\right) : p^*_T > 0.5 \end{cases} \quad (3)$$

where $E_{T,K}(0) = E_{T,K2}(0) = \sqrt{2s_2(v_1+v_2)NT_m/[(1+v_3)T_e]}$, $E^*_T = E^*_{T1}, p^*_T = p^*_{T1}$ in Case 1, and $E_{T,K}(0) = E_{T,K1}(0) = \sqrt{2s_2(1+s_5)VNT_m/[(s_3+s_4)T_e]}$, $E^*_T = E^*_{T2}, p^*_T = p^*_{T2}$ in Case 2. Please see Appendix S4 for the calculation of $E_{T,K}(0)$ in Case 1 and Case 2. If $p^*_T \leq 0.5$, the excess dead male eggs are additional provisioning for hatchling daughters; therefore, the maternal investment per hatchling of infected mothers exceeds the optimal value, because $p^*_{T,K}$ cannot be negative. Then, $p^*_{T,K}$ becomes 0, and $E^*_{T,K}$ becomes the optimal clutch size with male-killer infection at $P = 0$. If $p^*_T > 0.5$, infected as well as uninfected females can provide the optimal trophic egg proportion; in this case, the amount of trophic and dead male eggs consumed by each hatchling in an infected clutch is equal to the amount of trophic eggs consumed by each hatchling in an uninfected clutch.

Evolution of sibling cannibalism

Sibling cannibalism may affect the optimal clutch size and trophic egg proportion. The optimal clutch size and trophic egg proportion can be calculated by the optimization of mother's fitness, whereas the optimal probability of sibling cannibalism can be calculated by the optimization of cannibal offspring's fitness. Sibling cannibalism gives the advantage of additional food resources (viable eggs, not trophic egg) to cannibal larvae, but it also gives the disadvantage of falling victim to sibling cannibalism by other cannibal larvae. We constructed the model of sibling cannibalism, and calculated the condition of the evolution of sibling cannibalism (Appendix S6).

Empirical experiments

Study organisms

Harmonia yedoensis is a specialist ladybird that lives exclusively in pine trees and feeds mostly on the giant pine aphid *Cinara pini* Linnaeus in central Japan (Sasaji 1998). This prey species is nutritionally a relatively marginal food source for the development of ladybird larvae (Noriyuki and Osawa 2012). Moreover, *C. pini* is very mobile, so it is difficult for small ladybird hatchlings to hunt them efficiently (Noriyuki et al. 2011). To cope with this elusive prey, *H. yedoensis* mothers provide a relatively large proportion of trophic eggs to each clutch, even in the absence of the male-killer infection (Osawa and Ohashi 2008; Noriyuki et al. 2014). The consumption of trophic eggs greatly enhances the larvae's prey capture performance against *C. pini* and thus mitigates the risk of starvation in *H. yedoensis* hatchlings (Fig. S1; Noriyuki et al. 2011).

Experiment

In this study, the same data from the same individuals obtained by Noriyuki et al. (2014) were reanalysed as follows. In the previous study, females of *H. yedoensis* were collected at Kyoto, central Japan, in April and May 2011 and individually maintained in plastic Petri dishes at 25 °C and under a 16:8 h L:D photoperiod to obtain egg clutches. Every day, each Petri dish was replaced by a new one and a surplus of pea aphids *Acyrthosiphon pisum* was provided for food. Egg clutches were kept under the same laboratory conditions, and the numbers of eggs and hatchlings in each clutch from each female were counted. To standardize the effects of female age on reproductive output, we used first (up to) 15 clutches per female for the analysis. In addition, we examined the relationship between the order of oviposition and the clutch size and hatch rate to consider if female age affects the study results. Out of the 22 *H. yedoensis* females that laid sufficient number of egg clutches in their lifetime, approximately only half of the eggs in the clutches of 14 females hatched and they produced only daughters, as reported previously (Noriyuki et al. 2014). Diagnostic polymerase chain reaction analysis detected *Spiroplasma* spp. in these females (Noriyuki et al. 2014). By contrast, most of the eggs of the remaining eight females hatched, and the hatchlings included both sons and daughters. Furthermore, no *Spiroplasma* was detected by PCR analysis. Thus, in this study, we considered the 14 females of the former group to be infected with male-killing bacteria, and the eight females of the latter group to be uninfected. In addition, 12 infected females and 5 uninfected females were randomly chosen to examine egg size. The length (l) and width (w) of 10 eggs laid by each of the females were measured under a microscope as in the previous study (Noriyuki et al. 2014) and egg size was estimated as $lw2\pi/6$. In this study, we also measured body length of each female by an electronic caliper (Mitutoyo CD-15APX) to the nearest 0.01 mm, since the previous work in *Drosophila* demonstrated that male-killer infection affects female body size and then fecundity (Unckless and Jaenike 2012).

Statistics

We compared clutch size and egg size between infected and uninfected females by using the nested analysis of variance (nested ANOVA) program, with mother identity as a random effect, in the R software package (version 3.0.1, R Foundation for Statistical

Computing, Vienna, Austria). In clutches from uninfected females, unhatched eggs that were consumed by hatchlings were regarded as trophic eggs. In clutches from infected females, however, we were not able to distinguish between the killed male eggs and trophic eggs, because these two types of eggs did not differ in appearance and were consumed by the hatchlings in the same way. We therefore performed a Bayesian analysis with WinBUGS 1.4.3 (Spiegelhalter et al. 2003) to estimate the trophic egg proportion in infected and uninfected clutches. We assumed that the vertical transmission rate of male-killing bacteria was perfect (no male eggs in an infected clutch ever hatch). This assumption is valid because in the study population, no male offspring from infected lines developed to adulthood (Noriyuki et al. 2014). We also assumed that sex-ratio parity in each clutch. Thus, we calculated the hatch rates of uninfected and infected clutches as $1-q_i$ and $(1-q_n)/2$, respectively, where q_i and q_n are the trophic egg proportions in uninfected and infected clutches, respectively. Mother identity was treated as a random effect. The observed numbers of hatched eggs were assumed to follow binomial distributions and the logit link function was adopted. Prior distributions of parameters are usually used in Bayesian estimations, but we had no prior information about the trophic egg proportion or its inter-individual difference, so we adopted non-informative priors. Three independent chains of Markov chain Monte Carlo (MCMC) were calculated. The number of iterations of each MCMC was 100,000, the number of burn-ins was 20,000, and the thinning interval was 10. The convergence of each MCMC was determined by using the $\hat{R}$ (Gelman et al. 2014), smaller values of which indicate better convergence (minimum value, 1.0). When $\hat{R}$ was less than 1.1, we regarded the MCMC as having converged. The medians of the posterior sdistributions are regarded as the estimated values, and the 95 % Bayesian credible interval (BCI) is used as the indicator of the estimation error. The WinBUGS model used in the analysis is presented in Appendix S5.

Results

Model analysis

As hypothesized, the optimal clutch size was larger and the optimal trophic egg proportion was smaller for infected females than for uninfected females (Fig. 3, Fig. S2). These relationships were robust under various parameter settings, except for the special case in which the optimal clutch size was the same for both infected and uninfected females because the optimal trophic egg proportion of uninfected females $\left(p_T^*\right)$ exceeded 0.5 [Eq. (3)].

The difference in optimal clutch size between uninfected and infected females became larger as the food density (N), the feeding rate when no eggs were eaten (v_1), and the survival rate when no eggs were eaten (s_3) increased (Fig. 3a, c, Fig. S2*a, c*). This result indicates that the difference in clutch size became larger when the negative density effect was weak and the advantage of eating eggs was small.

The difference in the trophic egg proportion between uninfected and infected females became larger as the food density (N) and feeding rate (v_1) decreased (Fig. 3b, d). In Case 2, the difference became larger as the survival rate when no eggs were eaten decreased (s_3 in Fig. S2*d*). Difference between the optimal clutch size and the optimal trophic egg proportion in Case 2 was independent of the change of food density (N) (Fig. S2*b*).

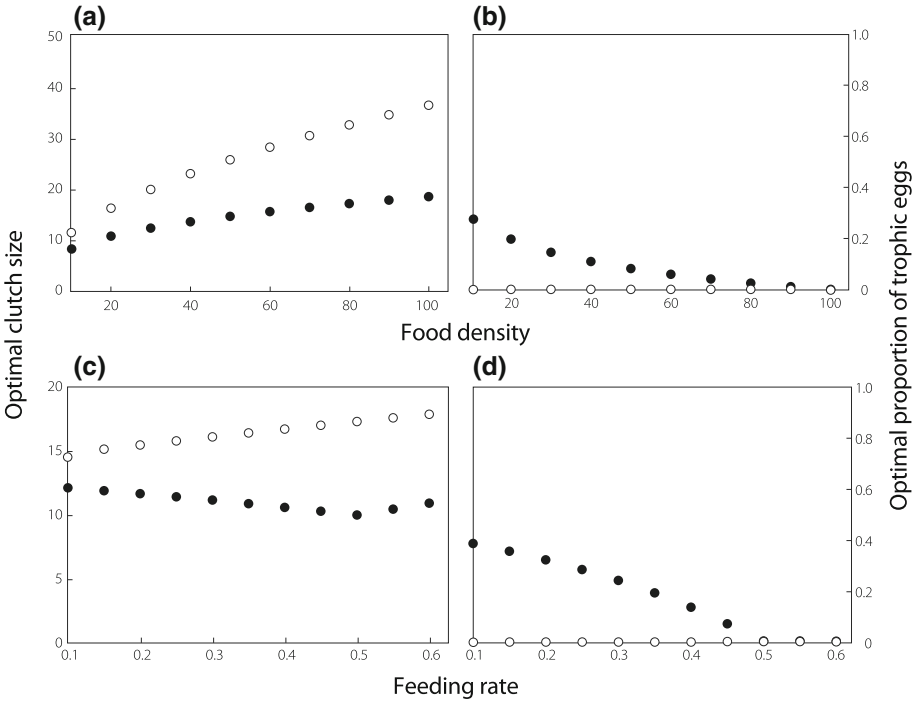

Fig. 3 Optimal clutch size and trophic egg proportion in infected (*open circles*) and uninfected individuals (*closed circles*) in Case 1. **a** Effect of food density (N) on optimal clutch size. Effects of s_2 and T_m/T_e on optimal clutch size are similar to that of N because they are related as s_2NT_m/T_e in Case 1 (see Appendix S2 and S4). **b** Effect of food density (N) on optimal trophic egg proportion. Effect of s_2 on trophic egg proportion is similar to that of N because they are related as s_2N in Case 1 (see Appendix S2 and S4). **c** Effect of feeding rate of offspring that eat no eggs (v_1) on optimal clutch size. **d** Effect of feeding rate of offspring that eat no eggs (v_1) on optimal trophic egg proportion. Other parameter settings are $s_2 = 1$, $N = 20$, $v_1 = 0.35$, $v_2 = 1$, $v_3 = 1$, and $T_m/T_e = 10$

In the model with antagonistic sibling interactions (Appendix S6), we observed that sibling cannibalism did not evolve after the optimization of clutch size and trophic egg proportion at least in the parameter range of this study (Fig. S3 in Appendix S6). Therefore, we compared the results of the simple model without sibling cannibalism (Fig. 3 and S2) with the experimental results.

Empirical analysis

Mean clutch size was significantly larger in infected females (mean ± SE = 15.241 ± 0.464, $n = 199$) than in uninfected females (12.880 ± 0.430, $n = 117$; nested ANOVA: fixed effect, $F_{1,\ 20} = 15.761$, $P < 0.001$; random effect, $F_{20,\ 294} = 6.414$, $P < 0.001$; Fig. 4a). Female body length was not significantly different between infected (mean ± SE = 7.219 ± 0.132 mm, $n = 14$) and uninfected females (7.335 ± 0.133 mm, $n = 8$; ANOVA: $F_{1,\ 20} = 0.331$, $P = 0.572$). Clutch size was significantly different between mothers in both infected (ANOVA, $F_{13,\ 184} = 8.049$, $P < 0.0001$) and uninfected females ($F_{7,\ 109} = 2.181$, $P < 0.05$; Fig. S4), but the order of oviposition had no consistent effect on clutch size in both infected and uninfected females

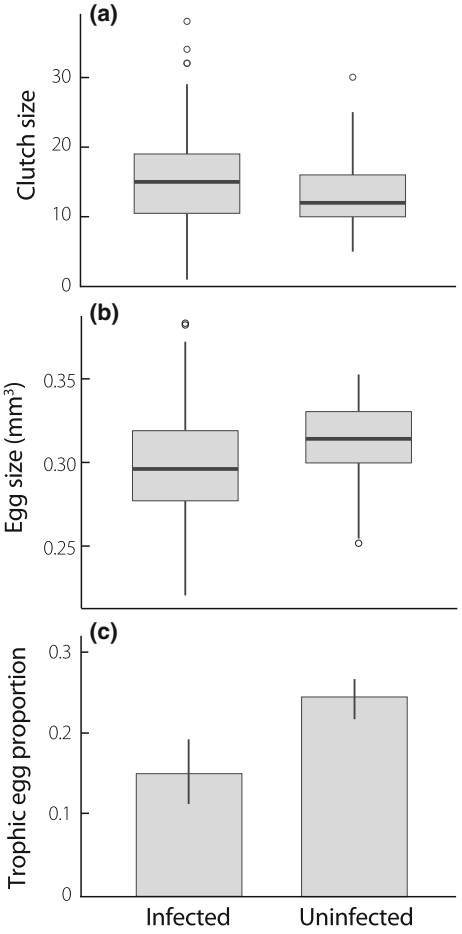

Fig. 4 **a** Clutch size, **b** egg size, and **c** trophic egg proportion of clutches produced by *Harmonia yedoensis* females infected or uninfected with male-killing bacteria. In **a** and **b**, *box*-and-*whisker plots* indicate the median (*bold line*), the 25th and 75th percentiles (*box edges*), the range (*whiskers*) and outliers, which are larger or smaller than 1.5 times the interquartile range from the box edge (*open circles*). In **c**, the mean and the 95 % Bayes credible interval (*bars*) are indicated

(Appendix S7, Table S1, Fig. S5). Egg size was significantly smaller in infected females (mean ± SE = 0.296 ± 0.001 mm^3, $n = 120$) than in uninfected females (0.310 ± 0.003 mm^3, $n = 50$; nested ANOVA: fixed effect, $F_{1,\ 15} = 27.07$, $P < 0.001$; random effect, $F_{15,\ 153} = 28.14$, $P < 0.001$; Fig. 4b). In the Bayesian model, the MCMC calculations converged in all three independent chains, and $\hat{R}$, the indicator of convergence, was less than 1.01 for all parameters. These results indicate that the model fits the data well. The estimated proportion of trophic eggs in uninfected (q_i) and infected females (q_n) was 0.242 (95 % BCI, 0.220–0.265) and 0.150 (95 % BCI, 0.114–0.192), respectively (Fig. 4c). Using these values, we calculated the odds ratio between q_i and q_n to be 0.555 (95 % BCI, 0.392–0.762).

Discussion

Our mathematical model predicted that host females infected with male-killing bacteria would produce a larger clutch and fewer trophic eggs (Fig. 3, Fig. S2). In line with this prediction, we showed experimentally that in *H. yedoensis* clutch size is larger, egg size is

smaller, and trophic egg proportion is lower in infected females than in uninfected females (Fig. 4). On the other hand, body size was not different between infected and uninfected females, suggesting no confounding effect of female body size on clutch size. Similarly, the order of oviposition had no consistent effects on clutch size and hatch rate in both infected and uninfected females (Appendix S7). The previous studies demonstrated the significant differences in adult body size, developmental time, and lifetime fecundity in ladybirds depending on the male-killer infection (Elnagdy et al. 2011, 2013). To our knowledge, however, this is the first time that empirical evidence supporting the predicted differences in clutch size, egg size, and trophic egg proportion with and without male-killer infection has been presented.

We showed theoretically that it is difficult to detect large differences in clutch size and the trophic egg proportion simultaneously between infected and uninfected lines, because these traits are predicted to exhibit opposite trends in response to environmental conditions. When the negative density effect is weak and hatchlings do not need to eat many eggs (i.e. good environmental conditions), clutch size should be about twice as large in infected line as in uninfected line, but the predicted difference in trophic egg proportion is less than 20 % (Fig. 3, Fig. S2). By contrast, when the offspring experience poor environmental conditions, the double difference in clutch size should not be expected but the trophic egg proportion should be similar or larger between infected and uninfected lines (Fig. 3, Fig. S2). In nature, *H. yedoensis*, hatchlings must catch a highly elusive aphid (Noriyuki et al. 2011); thus, in such an environment the difference in clutch size should be small. Therefore, the difference in mean clutch size between infected and uninfected mothers found experimentally in *H. yedoensis* (Fig. 4) is qualitatively consistent with our model prediction. It should be promising to examine the effects of environmental variables on clutch size and trophic egg proportion to quantitatively compare the model prediction and empirical data in the standardized condition.

Our mathematical model showed that host females that are infected with male-killing bacteria should provide no trophic eggs (Fig. 3, Fig. S2). Since bacterial manipulation drastically enhances the amount of maternal investment per offspring through resource reallocation from dead male embryos to female hatchlings, infected mothers should no longer add trophic eggs to the clutch. The experimental results, however, showed that infected *H. yedoensis* females produced a certain proportion of trophic eggs (Fig. 4b). This discrepancy between theory and data may be due to the peculiar population genetics that hampers the fixation of beneficial allele for infected individuals (Engelstädter and Hurst 2007; Jaenike 2007), indicating that incorporating population genetics can improve the model prediction in the further analysis. Moreover, sibling cannibalism might affect the study result (Fig. 4c) because in our experiment we could not strictly distinguish inviable trophic eggs from consumed viable eggs due to sibling cannibalism. Although our simple model did not predict the evolution of sibling cannibalism after the optimization of clutch size and trophic egg proportion (Appendix S6), offspring cannibalistic behavior can alter maternal decision making on trophic egg provisioning as predicted in the previous theoretical work (Perry and Roitberg 2005a). In addition, the proximate mechanisms of trophic egg production in ladybirds are largely unknown, and in *Harmonia* ladybirds in particular, the trophic egg proportion is highly variable even under standardized laboratory conditions (Majerus and Majerus 2000; Perry and Roitberg 2005b; Noriyuki et al. 2014). This phenotypic variability implies that it is difficult for ladybird mothers to control the trophic egg proportion precisely. However, Perry and Roitberg (2005b) have reported plastic adjustment of the trophic egg proportion in response to food availability. Moreover, the trophic egg proportion has been found to differ between *H. yedoensis* and *H. axyridis* under the

same laboratory conditions, indicating a genetic basis for this trait (Noriyuki et al. 2014). Further study is needed to clarify the mechanisms of trophic egg provisioning in order to understand their limitations and the potential for flexible adjustment of the host phenotype in the presence of male-killer infection.

Population genetics models predicted that beneficial alleles in infected individuals have a lower chance of fixation due to the peculiar inheritance pattern of nuclear alleles in male-killer-infected populations (Telschow et al. 2006; Engelstädter and Hurst 2007). By contrast, this study empirically demonstrated the variations in life-history traits depending on the infection of make-killing bacteria. Thus, it should be interesting to consider the mechanisms that allow host phenotypes to be modified as our model predicted. Gene flow from infected to uninfected individuals due to imperfect vertical transmission may enhance beneficial alleles to spread into the population (Jaenike 2007). Moreover, it may be possible that bacteria lead the manipulation of host phenotype even when nuclear genes in infected individuals are likely to disappear from the population. Further study is needed to untangle these mechanisms to clarify coevolutionary dynamics between bacterial endosymbionts and its host.

Acknowledgments This study was supported by a Grant-in-Aid for Scientific Research (No. 26840137) to S.N. and a Grant-in-Aid for JSPS Fellows (22.6920) to Y.S.O. from the Japan Society for the Promotion of Science.

Compliance with ethical standards

Conflict of interest This study was supported by a Grant-in-Aid for Scientific Research (No. 26840137) to S.N. and a Grant-in-Aid for JSPS Fellows (22.6920) to Y.S.O. from the Japan Society for the Promotion of Science.

Ethical standards All experiments using insects were carried out according to local ethical guidelines.

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