No evidence that presence of sexually transmitted infection selects for reduced mating rate in the two spot ladybird, *Adalia bipunctata*.

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Sexually transmitted infections (STIs) are common in animals and plants, and frequently impair individual fertility. Theory predicts that natural selection will favour behaviours that reduce the chance of acquiring a STI. We investigated whether an STI, *Coccipolipus hippodamiae* has selected for a reduced rate of remating by its host *Adalia bipunctata* as a mechanism to avoid exposure. We first demonstrated that rejection of mating by females did indeed reduce the chance of acquiring the mite. We then examined whether rejection rate and mating rate differed between ladybirds from mite-present and mite-absent populations when tested in a common environment. No differences in rejection intensity or remating propensity were observed between the two populations. We therefore conclude there is no evidence that STIs have driven the evolution of mating systems in this species.

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2   **reduced mating rate in the two spot ladybird, *Adalia bipunctata*.**

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Abstract

Sexually transmitted infections (STIs) are common in animals and plants, and frequently

impair individual fertility. Theory predicts that natural selection will favour behaviours that

reduce the chance of acquiring a STI. We investigated whether an STI, *Coccipolipus*

hippodamiae has selected for a reduced rate of remating by its host *Adalia bipunctata* as a

mechanism to avoid exposure. We first demonstrated that rejection of mating by females did

indeed reduce the chance of acquiring the mite. We then examined whether rejection rate and

mating rate differed between ladybirds from mite-present and mite-absent populations when

tested in a common environment. No differences in rejection intensity or remating propensity

were observed between the two populations. We therefore conclude there is no evidence that

STIs have driven the evolution of mating systems in this species.

1   INTRODUCTION

2   Sexually transmitted infections (STIs) can be defined as infections that are primarily

3   transmitted following sexual contact. Over 200 STIs have been identified to date and have

4   been discovered in 48 families and 27 orders of hosts (Lockhart et al. 1996). Hosts vary from

5   plants (e.g. the pollinator-transmitted anther smut *Ustilago violacea* which infects white

6   campion, *Silene alba* (Thrall et al. 1993)), through to mammals (e.g. *Trypanosoma*

7   *equiperdum* found in horses (Smith & Dobson 1992)). In the past, vertebrate STIs were the

8   most heavily studied and widely understood STIs, and insect STIs were somewhat neglected

9   (Lockhart et al. 1996; Lombardo 1998; Sheldon 1993; Smith & Dobson 1992). However in

10   more recent years, insect STIs have received increasing attention. Knell & Webberley (2004)

11   noted records of 73 species of STIs infecting approximately 182 species of insect. It is

12   notable that insect STIs are most commonly multicellular ectoparasites, such as mites, worms

13   and fungi, which contrasts with microbial STIs identified in vertebrates.

14   Most STIs have relatively small negative effects on host mortality, but tend to reduce

15   fecundity or sterilise the host (Lockhart et al. 1996). Natural selection should therefore favour

16   host traits that reduce the risk of infection. There are three possible behavioural routes to

17   reducing the chance of acquiring an STI. First, if female fertility is not limited by low

18   remating rates, exposure can be limited by mating with fewer partners. Theory predicts that

19   STI presence should select for an increase in female refusal to mate when courted (Boots &

20   Knell 2002; Kokko et al. 2002). Second, there is the possibility of rejection of infected

21   partners in favour of uninfected ones. Previous studies provide no evidence for such a choice

22   in either laboratory tests or natural populations (Abbot & Dill 2001; Nunn 2003; Webberley

23   et al. 2002). Finally, it has been postulated that some post-copulatory grooming processes,

24   and in cape ground squirrels, post-copulatory masturbation, may have evolved as a means of

25   preventing STI transmission (Hart et al. 1988; Nunn 2003; Waterman 2010).

1   The interaction between the two-spot ladybird, *Adalia bipunctata*, and its ectoparasitic mite

2   *Coccipolipus hippodamiae*, represents the best studied invertebrate-STI interaction. The mite

3   lives under the elytra of the beetle, and larval mites move between host individuals that are

4   copulating (Hurst et al. 1995). Mite infection in females is associated with a rapid loss of

5   fertility, such that acquiring an infection is very costly to females. The two-spot ladybird is a

6   promiscuous species where females mate once every 2-3 days in the wild (Haddrill et al.

7   2008). Where the mite is present, this promiscuity leads to an epidemic of this disease during

8   the spring/summer mating season, during which nearly all adult beetles become infected

9   (Ryder et al. 2014; Ryder et al. 2013; Webberley et al. 2006a).

10   The STI is thus both prevalent and highly costly to female hosts, creating a selection pressure

11   for direct avoidance of infected partners through mate choice, and indirect avoidance of mite

12   acquisition through reduced mating rate. Previous laboratory and field studies provided no

13   evidence that ladybirds discriminated against infected partners in mating decisions

14   (Webberley et al. 2002). However, the hypothesis that selection has acted to increase the

15   general tendency to reject matings has not been tested. One prediction of this hypothesis is

16   that rejection behaviour should be more intense, and mating rate lower, in ladybirds from

17   populations where the mite is present.

18   In this paper, we examine first whether rejection is efficient at preventing mite transfer, and

19   then test the hypothesis that ladybirds from populations in which the STI is present have been

20   selected for more intense rejection behaviour and lower mating rate, as a means of avoiding

21   infection. Our measures, which are made under standardized laboratory conditions, provide

22   no evidence that rejection behaviour or remating propensity differs between these

23   populations.

24

1 MATERIALS AND METHOD

3 *Experiment 1: Is rejection of mating by a female an efficent means of preventing transmission*

4 *of C. hippodamiae infection?*

5 Female and male ladybirds were collected from Stockholm in June/July 2011 and returned to

6 the laboratory. They were sexed and classified as being uninfected, latent infected or

7 infectious on the basis of absence of mites, presence of mites without infectious larval mites,

8 and presence of larval mites ready to transmit. Pairs comprising a single infectious male with

9 a focal uninfected female, and single infectious female with a focal uninfected male were

10 established in clean Petri dishes in the laboratory, and behaviour observed for 30 minutes.

11 Behaviour was scored as no interaction, rejected mating, and successful mating. Pairs that

12 mated were allowed to mate to completion before separation of the focal partner to a new

13 dish. The focal individual was then examined 24 hour later for the presence of larval mites.

14 The importance of focal host sex and mating/rejection on mite transfer was analysed with a

15 binomial GLM.

17 *Experiment 2: Do female beetles from populations that carry the STI show lower mating*

18 *rates and a greater likelihood of rejecting mating?*

19 *Adalia bipunctata* were collected from two locations c. 300 km apart in Sweden during

20 August 2012: Nässjö (57.7^{o}N, 14.7^{o} E) and Stockholm (59.3^{o}N, 18.1^{o}E). The Nässjö

21 population is free of mite infection (Webberley et al. 2006b), whereas there is an annual

22 epidemic of the infection in Stockholm, leading to nearly all beetles becoming infected

23 (Ryder et al. 2014; Ryder et al. 2013). Females from these populations were allowed to mate

24 with sympatric males, and progeny reared in the laboratory. This rearing was conducted

25 concurrently for both populations to standardize environment. The resulting adult ladybirds

1 were maintained in single sex dishes and allowed to mature for 30 days before experimental

2 analysis of rejection behaviour and mating rate. During this time, they were provided with an

3 ample supply of pea aphid food daily to ensure the beetles were in reproductive condition by

4 the time the experimental observations commenced. All behavioural observations occurred in

5 the absence of mites to avoid any direct impact of mites on the mating behaviour of their host

6 (although none have previously been observed: (Webberley et al. 2002))

8 Rejection behaviour and mating rate were analysed over daily mating trials carried out over a

9 five day period. 'Pools' of five females and five males were created for each population. In

10 each case males were from same population as females, but unrelated to them. Within each

11 pool, males and females were mixed and allowed to mate once three days before the

12 experiment. This was intended to reduce artefactual behaviour resulting from single sex

13 confinement. Subsequently, females from each pool were offered a male for 30 minutes at the

14 same time each day for a five day period, with each female being offered a different male

15 every day (see Table 1 for block design).

16 During each mating trial, each pair was placed in a clean Petri dish at 21°C for the duration of

17 the observation, and the presence of the following behaviour observed:

18   a) The number of interactions between male and female

19   b) The presence and duration of rejection behaviour during these interactions. Rejection

20   behaviour was categorised into different intensity levels; no rejection observed; mild

21   rejection (<1 minute); moderate rejection (1-5 minutes) and intense rejection (>5

22   minutes).

23   c) Whether interactions resulted in mating

1   From these measures, the likelihood of a female rejecting mating, the intensity of rejection,

2   and the probability of successful mating occurring were calculated.

3   Four replicate groups were used, resulting in 20 females being tested for each population.

1   RESULTS

3   *Experiment 1: Is rejection of mating by a female an efficent means of preventing transmission*

4   *of C. hippodamiae infection?*

5   Transmission rates from wild caught infectious male and female individuals to uninfected

6   partners with which they mated were high, with only one of 26 females not acquiring

7   infection during mating with an infectious male partner, and one of 35 males not acquiring

8   infection from an infectious female partner. In contrast, transmission was rare when mating

9   was rejected, with one of seven females acquiring an infection following rejection of the

10   infectious male, and one of three males acquiring infection having been rejected by an

11   infectious female. Statistical analysis revealed no evidence for an interaction term between

12   sex of infected host and mating/rejection behaviour on mite transfer probability. Statistical

13   analysis with the interaction term dropped revealed no effect of donor sex on transmission

14   probability (GLM factor host sex, p=0.288), but a significant effect of the factor

15   'rejected/mated' (GLM factor mated/rejected, p<0.0001). Thus rejection behaviour by the

16   female is protective against mite transfer both from an infected male, and additionally

17   prevents transmission to an uninfected male partner.

19   *Experiment 2: Do female beetles from populations that carry the STI show a greater*

20   *likelihood of rejecting mating and a lower mating rate?*

21   Mating was observed to be more common on day 1 than on other days in experiments

22   involving both Stockholm and Nässjö (Figure 1). Combining over blocks, and between

23   locations, mating was heterogeneous over the experiment (χ^2=16.042, df=4, p=0.003). This

24   heterogeneity is associated with high mating rates on day 1 (after 3 days without mating

25   activity); when day 1 is excluded, mating rates are homogenous over days 2-5 (χ^2=0.276,

1 df=3, p=0.964). Thus, in further analysis, day 1 mating is excluded, as the high mating rate

2 on this day is likely to be associated with experimentally induced lack of mating opportunity.

3 Of the 80 male-female interaction trials in each population observed after day 1, males

4 approached females for mating in 64 cases for both populations. Where interactions occurred,

5 most females exhibited some rejection behaviour in encounters after day 1, and this rejection

6 was prolonged in over half of cases in both populations. There was no evidence that females

7 from the two populations differed in the intensity of rejection behaviour following a male's

8 attempt to mate (χ^2=4.13, df=3, p=0.25) (Figure 2).

9 There was also no evidence for variation in overall propensity to mate between ladybirds

10 from Nässjö (mite free in nature) and Stockholm (mite present in nature) (Figure 1). Across

11 days 2-5, there was no evidence of an association between population and remating rate

12 (χ^2=0.627, df=1, p=0.428). We additionally reanalysed mating propensity to create a more

13 ecologically relevant statistic. The confined experiment of the Petri dish allows males the

14 ability to interact with female repeatedly, which is unlikely to occur in the field. An

15 'environmental' mating rate based on the result of the first interaction between male and

16 female only was therefore calculated, which discounted mating if this took more than five

17 minutes to achieve. The 'environmental' mating rate for Stockholm and Nässjö was half that

18 of the overall mating rate (Figure 3). Analysis indicates there was no evidence of association

19 between location and 'environmental' mating rate (χ^2=0.295, df=1, p=0.587).

20 DISCUSSION

21 Sexually transmitted infections are common in nature, and are frequently harmful to female

22 hosts (Lockhart et al. 1996). Models predict that the presence of STIs should therefore select

23 on female mating behaviour. Past work has failed to reveal any choice of mates associated

24 with STI avoidance (Abbot & Dill 2001; Nunn 2003; Webberley et al. 2002). However, there

1   has been no test of the hypothesis that selection will promote avoidance of STIs through

2   reducing propensity to remate (Boots & Knell 2002; Kokko et al. 2002). In this study, the

3   mating biology of ladybirds from two populations were compared, one from Stockholm

4   (where the STI is naturally present) and one from Nässjö (which is naturally uninfected). The

5   mating rate did not differ significantly between the two populations. Combined with previous

6   observations of lack of mate choice for uninfected partners, the data do not support the

7   hypothesis that STIs have selected on female mating behaviour in this species, despite

8   rejection of mating being partly effective at preventing STI transmission.

9   Failure to find a difference in remating rates between the two populations could have three

10 sources. First, there may be no difference. Second, there may be a difference but the effect

11 size is small (our experiment would not detect a 20% difference in mating rate). However, we

12 would note that mating rate was quantitatively higher in beetles from Stockholm (mite

13 present population) than Nässjö (mite absent). Third, the experiment may not be suitable to

14 detect a difference, for instances if it is removed from natural conditions. 'Naturalness' is

15 always a problem for laboratory study. Despite an experimental design that attempted to

16 replicate natural mating environment e.g. temperature, lighting, there were possibly critiques

17 of spatial confines, repeated interaction and ineffective behaviour. However, consideration of

18 the first interaction only did not alter the conclusion that mating/rejection did not vary

19 between populations. Thus, it is currently most parsimonious to conclude there are no fixed

20 biological differences in mating propensity between these two populations.

21 We are thus confident that the presence of a sterilizing STI that reaches high prevalence has

22 not led to the evolution of alteration of mating system between these two populations. Why

23 has an intuitive evolutionary path not been taken? One possibility is that a high mating rate is

24 required for female fertility, such that females who refuse to mate incur a cost. However,

25 *Adalia* females mated singly have equivalent fertility, measured over 20 days, to females

1   mated every two days (Haddrill et al. 2007). Thus, there is ample scope for a female's risk of

2   mite induced infertility to be reduced before sperm-depletion associated infertility is

3   observed. A second possibility is that local adaptation is not possible in this species.

4   However, the presence of variation in the frequency of colour pattern variants in this species

5   on equivalent spatial scales (Brakefield 1984) make us confident gene flow is not sufficient to

6   impede local adaptation. A third hypothesis is that selection to prevent STI does operate in

7   the way expected, but there are other factors differing between the populations that influence

8   mating rate evolution. It is possible that there is a counterbalancing selective force working in

9   opposition to the effect of the STI. The source of such selection is not obvious (the two

10 populations use similar habitat and have similar sex ratio), but such a hypothesis cannot be

11 ruled out. Finally, the prediction that STIs select for lower mating rate applies to female

12 hosts, in which there are smaller benefits to each additional mating, and in this species, higher

13 costs of infection (sterility). Selection on males is not expected to act in the same way, as

14 each mating provides significant fitness benefits, and the STI is only weakly costly to male

15 hosts (Ryder et al. 2007). If mating rate is determined by males, then the STI is less likely to

16 drive mating system evolution.

17 In summary, our experiment demonstrated rejection was efficient at preventing STI

18 transmission, but did not occur more commonly in beetles derived from populations where

19 the STI was common. This study, combined with previous analysis indicating STI infected

20 beetles were not disadvantaged in acquiring mates (Webberley et al. 2002), produces no

21 support for the hypothesis that mating behaviour evolves in response to the presence of a

22 sterilizing STI. An intriguing possibility is that STIs are most commonly observed in species

23 in which evolution to resist STI transmission is inhibited.

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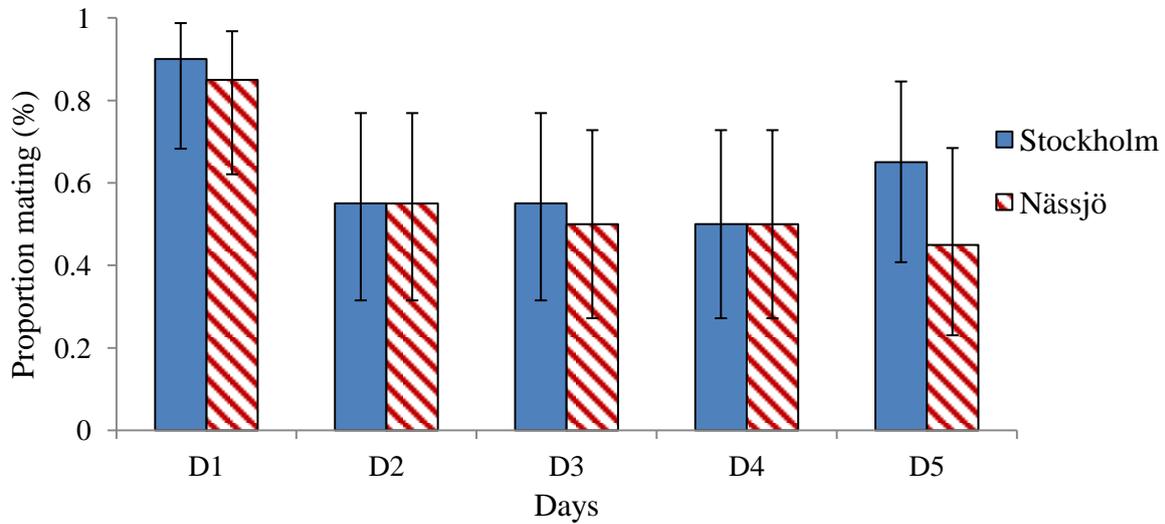
1 Table 1: Five day experimental block design of sympatric matings between Stockholm

2 (SF1=Stockholm Female 1, SM1=Stockholm Male 1 etc) and Nässjö (NF1= Nässjö Female

3 1, NM1= Nässjö Male 1 etc.) individuals. Numbers in the matrix indicate day of mating.

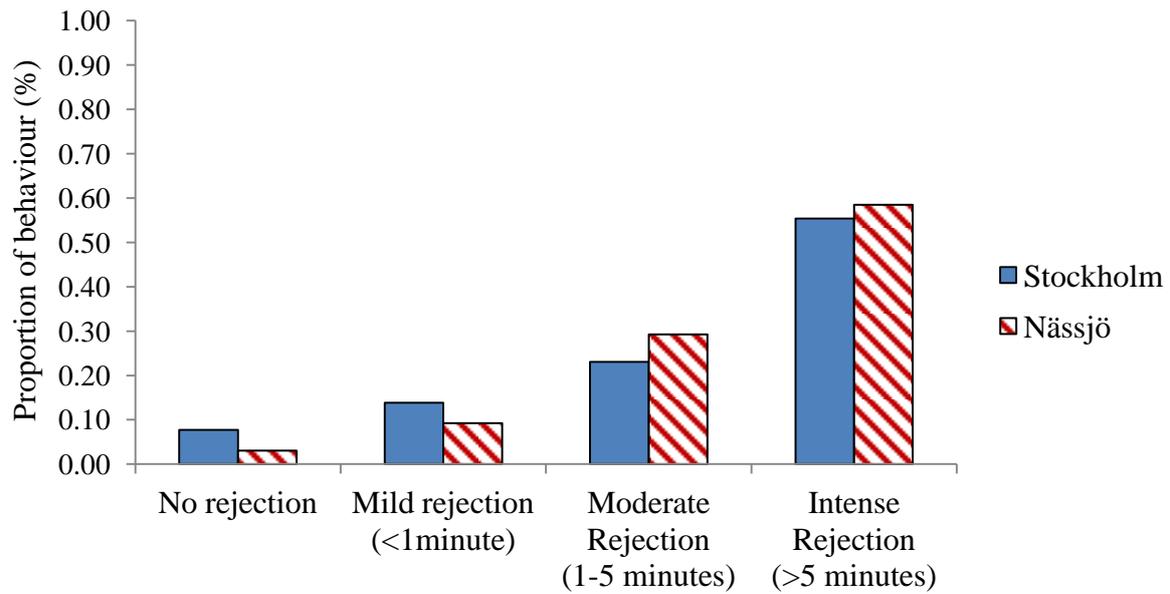
	SF1	SF2	SF3	SF4	SF5
SM1	5	4	3	2	1
SM2	1	5	4	3	2
SM3	2	1	5	4	3
SM4	3	2	1	5	4
SM5	4	3	2	1	5

	NF1	NF2	NF3	NF4	NF5
NM1	5	4	3	2	1
NM2	1	5	4	3	2
NM3	2	1	5	4	3
NM4	3	2	1	5	4
NM5	4	3	2	1	5

3   Figure 1: Proportion of pairs that mated each day during 30 minute period from Stockholm

4   (Blue, STI naturally present in nature, though absent in the experiment) and Nässjö (Hatched

5   Red, no STI). N=20 for all days, the combined results from four blocks. Error bars for

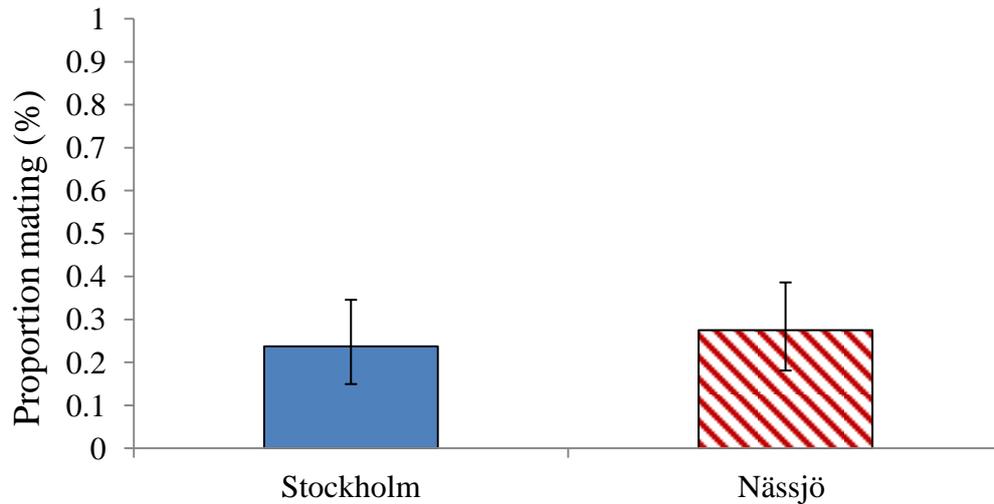
6   proportionate data represent binomial sampling intervals calculated using the Clopper –

7   Pearson (1934) method.

2 Figure 2: Proportion of different intensities of rejection behaviour (No rejection, mild

3 rejection (<1minute), moderate rejection (1-5minutes), intense rejection (>5minutes))

4 observed from Stockholm (Blue, STI naturally present, though absent in the laboratory) and

5 Nässjö (Hatched Red, no STI) females during 30 minute period experiments over days 2-5.

6 N=64 for both populations.

2   Figure 3: 'Environmental' mating rate P for Stockholm (Blue, STI naturally present, absent

3   in the laboratory) and Nässjö (Hatched Red, no STI) ladybirds over days 2-5. A pair was

4   considered to have mated only if the first interaction between male and female led to mating.

5   N= 20 female beetles, 80 interactions, for both populations.