Genetica (2018) 146:171–178
https://doi.org/10.1007/s10709-018-0009-z

ORIGINAL PAPER

Testing the effect of paraquat exposure on genomic recombination rates in queens of the western honey bee, *Apis mellifera*

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Received: 3 September 2017 / Accepted: 30 January 2018 / Published online: 3 February 2018

Abstract
The rate of genomic recombination displays evolutionary plasticity and can even vary in response to environmental factors. The western honey bee (*Apis mellifera* L.) has an extremely high genomic recombination rate but the mechanistic basis for this genome-wide upregulation is not understood. Based on the hypothesis that meiotic recombination and DNA damage repair share common mechanisms in honey bees as in other organisms, we predicted that oxidative stress leads to an increase in recombination rate in honey bees. To test this prediction, we subjected honey bee queens to oxidative stress by paraquat injection and measured the rates of genomic recombination in select genome intervals of offspring produced before and after injection. The evaluation of 26 genome intervals in a total of over 1750 offspring of 11 queens by microsatellite genotyping revealed several significant effects but no overall evidence for a mechanistic link between oxidative stress and increased recombination was found. The results weaken the notion that DNA repair enzymes have a regulatory function in the high rate of meiotic recombination of honey bees, but they do not provide evidence against functional overlap between meiotic recombination and DNA damage repair in honey bees and more mechanistic studies are needed.

Keywords Meiotic recombination · Oxidative stress · *Apis mellifera* · Genome evolution · Social evolution · Stress response

Introduction

The western honey bee (*Apis mellifera* L.) is eusocial and lives in colonies containing three distinct castes: drones, workers, and queens. A colony may have hundreds of haploid males (=drones) that develop from unfertilized eggs with only one set of 16 chromosomes. A typical colony also has thousands of female workers and one queen that develop from fertilized eggs and are thus diploid. Queens mate at the beginning of adult life and subsequently serve as sole reproductives in their colonies, living an extraordinarily long life (Page and Peng 2001). Workers perform all other colony functions, displaying a cooperative, age-based division of labor (Winston 1987). Social evolution has led to strongly divergent selection pressures on the genome of the honey bee, based on the different functions of these castes. The history of strong, divergent selection might explain why honey bees have evolved the highest recombination rate among Metazoans (Beye et al. 2006; Kent et al. 2012; Kent and Zayed 2013; Liu et al. 2015; Ross et al. 2015; Wallberg et al. 2015; Rueppell et al. 2016).

Honey bees are one striking example of the evolutionary dynamics of genome-wide recombination rates. While the high rate is preserved within this genus (Rueppell et al. 2016), the recombination rates of 20 cM/Mb and > 2 crossovers per chromosome arm are much higher than those of most other metazoan species (Beye et al. 2006; Wilfert et al. 2007). Typically, only one or two crossover events per homologous chromatid pair occur and recombination rates vary largely as a function of genome size (Lynch 2006). The high recombination rates in honey bees raise the important question: How is this high level of chromosomal exchange not selected against due to potential ectopic recombination or non-disjunction of the pairing chromosomes? Other social insect species in the order Hymenoptera also have elevated recombination rates (Beye et al. 2006; Wilfert et al. 2007) and the evolutionary lability of genomic recombination rates

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has become apparent in other taxa as well (Dumont and Payseur 2011): within species and genomes, local recombination rates also vary profoundly (Kong et al. 2014; Ross et al. 2015; Adrian et al. 2016; Hunter et al. 2016).

In honey bees nothing is known about the recombination machinery that explains local variation in recombination and enables the high recombination rates. In contrast, substantial information on the mechanisms and genetic control of meiotic recombination exists in model organisms. PRDM9, one of the influential DNA-binding proteins, accounts for a large portion of the fine-scale regulation of recombination in several vertebrate genomes (Baudat et al. 2013), but numerous other genes have also been involved in the process of meiotic recombination in yeast, mouse, and other models (Kohl and Sekelsky 2013). Many of these genes, such as Rad51, Hop2, and BRCA2 are also involved in DNA damage repair (Li and Heyer 2008). Although no direct studies of the mechanisms of meiotic recombination or DNA damage repair exist in honey bees, their genome contains homologs to numerous genes that have been associated with these two processes (Elsik et al. 2014). Thus, it is reasonable to assume that the mechanisms of DNA damage repair and meiotic recombination have been largely conserved in honey bees, despite the extraordinarily high rate of recombination.

In all species, meiotic recombination is strictly limited to the formation of germ cells and is initiated via DNA double strand break (DSB) formation by SPO11 (Keeney 2008). DSB and other DNA damage accumulates continuously in most cells requiring constant DNA repair. Consequently, the majority of the DNA damage repair genes are constitutively expressed (Slupphaug et al. 2003). However, an increase in DNA damage can induce an upregulation of DNA damage repair pathways to protect genomic integrity (Mannuss et al. 2012). DNA damage and its repair can be caused by oxidative stress that is derived either from cell-endogenous processes, such as energy production in the mitochondria, or from exogenous sources, such as radiation or xenobiotics (Cadet et al. 2010).

A frequently-used experimental tool to induce oxidative damage is the pesticide *N,N′*-dimethyl-4,4′-bipyridinium, with the common name paraquat (Bus and Gibson 1984; Cooke et al. 2003; Mannuss et al. 2012; Tokunaga et al. 1997). Paraquat triggers redox cycling reactions inside the cell that produce free radicals that can attack DNA in various forms (Cooke et al. 2003) and paraquat-induced oxidative damage affects DNA integrity in a number of organisms (Tokunaga et al. 1997; Dinis-Oliveira et al. 2008). Honey bees are susceptible to paraquat to varying degrees: The egg-yolk protein vitellogenin, produced in the fat body cells of the abdomen, displays anti-oxidant properties and natural variation in vitellogenin titers among workers correlates with paraquat resistance (Seehuus et al. 2006). Sublethal topical doses applied to newly emerged workers have little effect (Rueppell et al. 2017) while a 10× lower doses of paraquat injected into mature drones kills the majority within a few days (Li-Byarlay et al. 2016). Due to their reproductive specialization, honey bee queens have vitellogenin titers that are much higher than in workers (Corona et al. 2007) and they overexpress other protective genes (Aamodt 2009), although some antioxidant genes are upregulated in workers relative to queens (Corona et al. 2005).

Based on the considerable genetic overlap between meiotic recombination and DNA damage repair in most well-studied models and the hypotheses that oxidative damage may be an evolutionary and mechanistic origin of meiosis (Bernstein et al. 2012; Hörandl and Hadacek 2013), we hypothesized that this overlap also exists in honey bees. Assuming that DNA damage repair pathways can also be induced in honey bees by free radicals, we predicted that sublethal paraquat injections into honey bee queens would increase rates of meiotic recombination. We tested this prediction by establishing a sublethal paraquat injection protocol for honey bee queens and genotyping offspring produced by multiple queens before and after paraquat injection. Our results demonstrate significant effects of the paraquat injection on recombination in individual queens but fail overall to support our prediction. Thus, more direct studies are needed to understand the mechanistic causes of the extraordinarily high recombination rate of honey bees.

Methods and materials

Sample preparation and pre-treatment collection

Experimental queens were produced by grafting first-instar larvae from various hives in the UNCG apiary into plastic queen cell cups that were then transferred into a queen rearing hive (Laidlaw and Page 1997). Capped queen cells were moved into small, queenless nuclear hives for emergence, mating, and subsequent establishment as mature egg-layers. Additionally, a few established queens from other sources were incorporated into the study. All hives containing experimental queens were maintained in the UNCG apiary throughout the experiment and therefore all queens were treated as one experimental group. Once the queens were established (as evidenced by a regular egg-laying pattern, typically 2–4 weeks after emergence), 200 larvae were collected from each queen prior to paraquat injection, and these samples were frozen at − 80 °C for later processing.

Paraquat treatment and post-treatment collection

Based on previous results (Corona et al. 2007) we conducted preliminary trials to determine the optimal sublethal dose of paraquat injection, after oral administration was ruled out

due to irregular feeding behavior of independent queens. An injection of 1 µL of a 10 µg/µL paraquat solution buffered with PBS was chosen to exert a severe oxidative stress that still allowed enough queens to survive and produce offspring for several weeks. Injections were made with a glass capillary needle laterally between the 2nd and 3rd abdominal segments of CO_2-anesthetized queens in order to introduce the paraquat as close to the ovaries as possible without severe injury. Similar injections with PBS buffer did not cause any mortality in several test queens. After paraquat injection, 26 queens were returned to their hives to resume egg-laying. Fifteen of these queens died after treatment, many with a darkened abdomen, suggesting paraquat-mediated cell death (Bus and Gibson 1984). From each of the 11 surviving queens, 200 post-injection samples were collected as brood derived from eggs that were laid 3 weeks after injection to ensure that the corresponding meiosis had occurred with ample time for the treatment to take effect.

DNA extraction and genotyping

DNA was extracted from samples using a previously established Chelex™ extraction protocol in 96-well format, adding 150 µL of a 5% Chelex™ solution and 5 µL of proteinase K solution (10 mg/mL) to a thin slice of tissue. After incubation at 55 °C for 1 h, DNA concentration was determined using a Nanodrop™ spectrophotometer, and samples were diluted to 10 ng/µL and stored at − 80 °C for subsequent genotyping by PCR. Pairs of microsatellite loci on chromosome 3 with intermediate linkage distances based on a previous linkage map (Solignac et al. 2007) were chosen (Table 1). Chromosome three is most representative for the honey bee genome because it is close to the average in terms of size (Chr.3: 13.2 Mbp vs 13.7 Mbp chromosomal average), annotated gene number (764 vs 782), and GC content (35.7 vs 35.4%). Based on previous protocols (Meznar et al. 2010), each 15 µL PCR contained 1 µL of diluted template DNA, 1.5 µL dNTP mix (2 mM), 0.75 µL forward and 0.75 µL reverse primer solution (10 µM), 1.5 µL PCR buffer (10×), and 0.05 µL Taq DNA polymerase (5U/µL). PCR conditions were adopted from (Meznar et al. 2010) and annealing temperatures for each locus optimized using a gradient cycler (range 45–60 °C). Microsatellite loci were screened for maternal heterozygosity and different primer pairs were used to genotype sample sets accordingly. Figure 1 depicts these loci with their position in a previously produced linkage map (Solignac et al. 2007). The total genome interval spans 3.5 Mbp and 80 cM, corresponding to approximately 1.5% of the physical and 2% of the genetic map, respectively. Each PCR product was mixed with 2 µL of stop buffer, denatured at 95 °C for 3 min, and sized by 6% polyacrylamide gel electrophoresis on a Licor DNA Analyzer 4300™. Maternal allele sizes were determined in all pre- and post-injection samples to quantify recombination rates for adjacent markers. Samples that yielded no or ambiguous genotyping results were excluded from data analysis. These missing data were caused by the following two problems: (1) in many cases, a paternal allele that equaled one of the maternal alleles prevented a determination which of the two maternal alleles was present in the offspring. (2) In several

Table 1 Microsatellite loci between which recombination rates were measured

Locus	Primer sequences	Physical location (bp)	Recombination position (cM)
AC149	Forward: CGAGTCGAAACCTTTACACC	8,591,065–8,591,179	191.3
	Reverse: CGATGAACTTAATTGGCTCC		
UN157T	Forward: AACCTCGAAAAAGCGTCTGG	9,193,051–9,193,158	201.7
	Reverse: GCGTCCCACCTACTCTCAGC		
K0353	Forward: TTGTTGACATCGTGCGCC	9,785,478–9,785,660	215.8
	Reverse: CGTCATCGACATTCGTTTCG		
K0311	Forward: AATTCCAGGCCAATGAATCA	9,965,958–9,966,254	221.3
	Reverse: TGATAAGATTTCGCAACGATTC		
K0351	Forward: AGTGAAAATACGACAAAGCATCG	10,537,446–10,537,670	239
	Reverse: CGAATCGAAACGAGCAT		
AT066	Forward: CGTCCGTTATCCACTCGG	11,525,778–11,526,028	248.7
	Reverse: GAGGAGCAGTTTCGCGAC		
UN295	Forward: CTCCATCGTCTACGGGCAG	11,685,349–11,685,505	265.6
	Reverse: GGTTGGCGCCACTGAGTAG		
SV196	Forward: TCACGCAAAGTCAAACGAGC	12,057,293–12,057,464	270.3
	Reverse: TTTCGAACCGTTTCGACAAAC		

Locus names are given according to (Solignac et al. 2007). Primer sequences, physical location, and genetic linkage position according to (Solignac et al. 2007) are listed

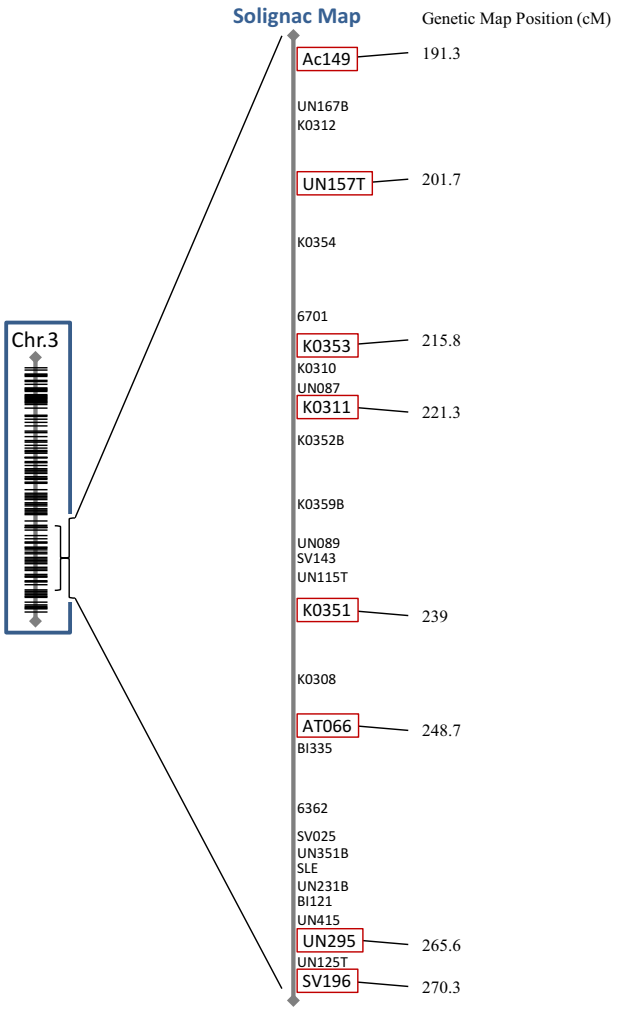

Fig. 1 Genetic position of studied loci. All loci were located on chromosome 3, an intermediate-sized acrocentric chromosome. The area of study was away from centromeric and telomeric regions and is enlarged to indicate individual loci (red boxes) with their genetic location (in cM)

other cases the quality of PCR products was insufficient to accurately genotype individual offspring despite a second attempt of scoring the corresponding gel and an additional independent replicate PCR.

Statistical analysis

Recombination in each interval was quantified before and after paraquat treatment by analyzing the multi-locus genotypes of the flanking genetic markers. Based on the overall frequencies of the four possible marker combinations (two loci × two alleles, e.g., "AB", "aB","bA", and "ab"), the rarer of the two complementary genotype pairs ("AB" and "ab" vs. "Ab" and "aB") was determined to be recombinant under the theoretical assumption that the frequency of recombinants between two markers can never exceed 50%. Based on this designation, the number of recombinant and non-recombinant allele combinations were calculated separately before and after paraquat exposure for each genome interval and queen. The number of recombinant vs non-recombinant offspring was then compared between pre- and post-injection samples for each interval, queen, and overall by Fisher's exact tests. Results were also assessed for consistent directional change across all queens with a simple sign test.

Results

Adjacent microsatellites markers bordering 1–5 genome intervals from eleven experimental queens were successfully genotyped to allow a comparison of recombination rates in these genome intervals between pre- and post-injection samples. Across all queens, 26 intervals were analyzed in total. Combining these 26 intervals for an overall test, the ratio of recombinant to non-recombinant genotypes was higher after paraquat injection (576 recombinants: 1097 non-recombinants) than before injection (602:1273) but the difference was not significant (Fisher's exact test: one-tailed $p = 0.076$). Out of 26 intervals the ratio of recombinant to non-recombinant genotypes increased in 14 intervals and decreased in 12 intervals after injection, also indicating no overall difference (sign-test: one-tailed $p = 0.423$). Four of the 14 increases in recombinant genotypes of individual intervals and one of the 12 decreases were significant before Bonferroni correction and two of the increases in recombinant genotypes remained significant after Bonferroni correction (Table 2). Summed over the measured intervals of each queen, seven queens exhibited higher ratios of recombinant to non-recombinant genotypes after paraquat injection than before but the difference was only significant in two individuals as determined by a sign test (Fig. 2).

Discussion

The overall results do not support our prediction that oxidative stress increases meiotic recombination rates in honey bees. This prediction was formulated based on the hypothesis that DNA repair and meiotic recombination share common mechanisms that can be upregulated by oxidative damage to the genome. Additional, more general hypotheses that postulate a link between oxidative stress and meiotic recombination have been formulated based on mechanistic and evolutionary arguments (Bernstein et al. 2012; Hörandl and Hadacek 2013). Our study fails to support these hypotheses overall. However, the data indicated a trend towards higher recombination rates after paraquat injection: In addition to the slight overall increase in recombination, a two individual queens showed a significant increase in the ratio

Table 2 Interval-specific changes in recombination rate after paraquat stress

Queen ID	Interval	Pre-paraquat recombination rate	Post-paraquat recombination rate	Significance (p-value)
1	UN157T–K0311	22/51	6/18	0.5807
1	K0311–K0351	21/44	8/33	0.0565
1	**K0351–AT066**	**24/104**	**38/79**	**0.0005**
1	AT066–SV196	45/131	33/110	0.4925
1	K0311–AT066	24/57	7/21	0.6045
2	K0353–AT066	14/53	16/34	0.0649
3	UN157T–K0311	11/87	20/95	0.1676
4	K0353–K0351	11/29	24/42	0.1488
4	K0351–AT066	3/17	19/48	0.0859
5	UN157T–K0311	26/94	34/111	0.7582
5	K0311–AT066	12/53	25/97	0.6978
6	AC149–K0311	19/60	40/115	0.7379
6	K0311–AT066	23/51	14/46	0.1497
6	AT066–SV196	26/67	25/57	0.5878
7	UN157T–K0311	23/136	15/107	0.5961
7	K0311–AT066	33/86	22/80	0.1425
8	**UN157T–K0311**	**12/84**	**27/57**	**0.0002**
8	**K0311–AT066**	**18/56**	**29/50**	**0.0107**
8	AT066–SV196	32/73	29/73	0.7373
9	AC149–K0351	31/74	24/43	0.1798
9	**K0351–SV196**	**37/79**	**14/49**	**0.0436**
10	UN157T–K0311	9/30	16/82	0.3054
10	K0311–K0351	24/94	14/85	0.1482
10	**K0351–UN295**	**38/88**	**49/73**	**0.0027**
11	UN157T–K0311	33/89	12/33	0.9999
11	K0311–AT066	31/88	16/35	0.3085

Different genome intervals between adjacent microsatellite loci were measured in different queens, as indicated in the first two columns. Numbers in the third and fourth columns indicate the recombinant over the total individuals genotyped for each specific test. Significant changes (uncorrected $p < 0.05$) between pre- and post-paraquat exposure of the queens are indicated in bold

of recombinant to non-recombinant genotypes after paraquat injection, and several increases were also significant in a few specific genome intervals for two other queens.

The heterogeneity of results, specifically the inter-individual variation, suggests that only a subset of the queens may have suffered from oxidative DNA damage sufficiently to influence the recombination rates during egg production. Honey bee queens have hundreds of post-meiotic eggs maturing in their ovaries and even though they can lay up to 2000 eggs per day (Winston 1987), it is unclear how much time passes between meiosis and egg-laying in any given individual. Eggs that gave rise to the sampled post-injection offspring were laid 3 weeks after the actual injection, which might have been mistimed for measuring the effect of induced DNA repair mechanisms. In contrast to *Drosophila* (Singh et al. 2015), too little is known about the timing of the cellular and molecular processes of egg maturation in honey bees to select a narrow time window for assessing induced changes to meiotic recombination. Given the long ovarioles of honey bee queens, the selected 3-week period appears an optimal choice. However, egg-laying rates during these 3 weeks strongly varied, which also could explain the variation in results among queens. It is also important to note that we only evaluated a small fraction of the total genome and that the size of the studied genomic interval varied among queens due to the availability of polymorphic markers that could be successfully genotyped. Thus, the heterogeneity among queens may also be explained by methodological limitations.

However, the overall results may also indicate that there was no effect of paraquat administration on meiotic recombination rates. It cannot be excluded that the paraquat dose was too low, although about half of all experimental queens died from the treatment before resuming egg laying. Their deaths were presumably due to the paraquat because comparable non-injected queens maintained in the same apiary during the experimental period did not exhibit any mortality. Our "before and after treatment" design instead of comparing

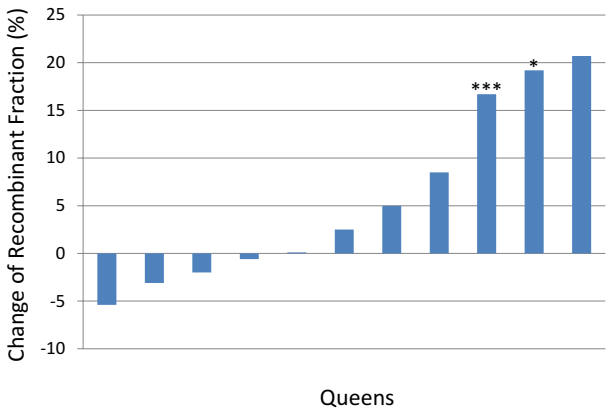

Fig. 2 Significant heterogeneity in treatment effects among queens. For all individual queens the relative change (expressed as %) of recombination rates after paraquat injection is plotted. No consistent effect of paraquat injection on recombination rates across all queens was found, although across all intervals studied, more queens showed the predicted increase of recombination rate. In two queens this effect was significant (*$p < 0.05$, ***$p < 0.001$) although one other queen exhibited an even stronger increase

treatment and control groups allowed us to account for natural inter-individual variation in recombination rates. The variation among queens in the initial measurements of recombination before treatment validates this strategy. We cannot exclude the possibility that the observed changes in recombination rates within individuals were due to experimental handling or aging of the queens during the experiment. However, all conceivable precautions were taken to minimize handling stress and these routine procedures seem negligible compared to the paraquat-induced toxicity. Moreover, our experimental period was very short compared to the overall lifespan of honey bee queens (Rueppell et al. 2004), which makes aging effects on recombination an unlikely explanation of our results.

A serious methodological limitation was our inability to quantify DNA damage in the remaining experimental queens because most of these queens had also died while producing the post-injection offspring before they could be sampled. Again their high mortality also suggests substantial oxidative damage but we lack a definite confirmation. Meiotic recombination rates may also have been unaffected due to specific protective mechanisms of the germarium in which meiosis occurs. Paraquat was injected near the internal location of the ovaries to achieve maximum impact, but we cannot exclude the possibility that the stem cells undergoing meiosis are particularly well protected against environmental insults. Mosquito ovaries have specific antioxidant protection (Sim and Denlinger 2011) and the high vitellogenin titer in honey bee queen abdomens also functions as an antioxidant (Corona et al. 2007). Double-strand breaks induced by oxidative damage may also be repaired through mechanisms that are favoring recombination pathways that are resolved through non-crossover events (Hörandl and Hadacek 2013). Non-crossover events would not have been detected in our study because our genetic markers were spaced widely to optimize the statistical power of our analyses.

To our knowledge, this experiment was the first study to test directly the connection between induced DNA damage and meiotic recombination rates at the organismal level, although molecular studies relating specific genes to DNA repair and meiotic recombination exist in select model organisms (Ghabrial et al. 1998; Vispé et al. 1998; Schuermann et al. 2005; Neale and Keeney 2006). Nevertheless, it is possible that meiosis-specific factors (Blanton et al. 2005) constitute the rate-limiting step whose modification has led to the recombination rates of all honey bees (Rueppell et al. 2016). Without standard genetic tools, the mechanistic basis of the genome-wide upregulation of recombination rates in the honey bees is difficult to explore. Our study was designed to provide some data to start to build a mechanistic model and alternative approaches to mechanistically explain the exceptional recombination rates of honey bees will have to pursued. Although some of our data suggest a possible connection between oxidative stress-induced DNA repair and meiotic recombination, this study was unsuccessful to provide unequivocal support for this hypothesis. In any case, the goal of a mechanistic understanding of the evolution towards elevated recombination rates remains important, particularly because studies that analyze the patterns of recombination across the genome to find adaptive explanations (Bessoltane et al. 2012; Kent et al. 2012; Liu et al. 2015; Ross et al. 2015; Wallberg et al. 2015) have not resulted in a satisfactory consensus so far.

Acknowledgements We would like to thank all members of the Social Insect Lab at UNCG for their encouragement and discussion. Financial support for this study was provided by a NIGMS grant to OR (R15GM102753) and the Army Research Office (W911NF1520045).

Funding The funder had not role in experimental design, performance, analysis, or interpretation of this study.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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