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Original Article

Female transcriptomic response to male genetic and nongenetic ejaculate variation

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Postcopulatory variation in reproductive success is fundamental for sexual selection. Because evolutionary change is impossible without a heritable basis for variation, the study of postcopulatory variation has mainly focused on genetic differences between males, that is, the effect of sperm competition or differential female responses toward male genotypes (cryptic female choice). The role of environmental components in shaping postcopulatory variation in reproductive success is well known, for example, in the form of damaging lifestyle effects on sperm, but their effect on eliciting female responses has rarely been tested, as has its relative significance compared with male genotypic effects. Here we provide such a test in bedbugs, a species where cryptic female choice has been hypothesized to be directed toward specific sperm genotypes. We measured female transcriptomic responses after experimentally controlling the male genetic and environmental component of the ejaculate. For identical female genetic background and identical male age at mating, we analyzed female gene expression in response to insemination with sperm of 3 different inbred populations (genotypes), each exposed to 1 of 2 environmental treatments (sperm storage duration in the male). Females responded mainly to environmental variation: >15 times more genes were differentially expressed, including stress response genes, compared with male genotypic variation. Our results suggest that postcopulatory natural selection exists and plays a significant role in the evolution and diversification of reproductive traits. Our results add complexity to testing the cryptic female choice hypothesis and show that nongenetic ejaculate effects are an important but underappreciated source of variation in biology.

Key words: ***Cimex lectularius*, fertility, genotype by environment interaction, sperm ecology, sperm senescence.**

INTRODUCTION

Evolutionary change is caused by variation among individuals in heritable components of reproductive success. Studies of natural selection thereby largely focus on the variation caused by differential survival in different environments, whereas sexual selection research focuses on differential reproductive success per se. The latter can occur before or after mating via pre- or postcopulatory selection. Postcopulatory variation arises from differences between male genotypes (sperm competition—Parker 1970; Simmons 2001) but is also shaped by interaction effects with female genotypes (Clark et al. 1999), including cryptic female choice of sperm from vital, courtship-stimulating or compatible males (Eberhard 1996). Cryptic female choice is defined as the postcopulatory processes involved in the differential use of sperm genotypes for fertilization by the female (Eberhard 1996). Females select specific and favorable genotypes through behavioral, morphological, and physiological mechanisms (Eberhard 2009) and therefore might involve differential gene expression (Mank et al. 2013). The importance of female postcopulatory responses to sperm as a selective pressure on males manifests in the finding that sperm traits coevolve with females (Miller and Pitnick 2002). The mechanistic basis of how certain genetic variation in sperm or ejaculate characteristics, such as sperm length, cause the observed differences between male genotypes is, however, not well understood (Birkhead et al. 2009, but see Fry and Wilkinson 2004; Pattarini et al. 2006; Manier et al. 2010) and related to both genetic and nongenetic variation in sperm function (Snook 2005; Reinhardt 2007; Pizzari et al. 2008a).

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Sperm function is the quantitative and qualitative expression of the sperm phenotype. Just as phenotypes of individuals, the sperm phenotype can be partitioned into a male genetic and an environmental component (Parker and Pizzari 2010), leaving aside genetic differences between sperm of the same male (the sperm genotype *sensu stricto*). In addition to male genotypic sources, variation in reproductive success can also arise when environmental factors affect the sperm phenotype during sperm production and storage in the male, or during sperm storage by the female. For example, in reproductive medicine environmental factors, so-called lifestyle effects, such as diet, smoking habits are known to alter a variety of biochemical characteristics of sperm and lead to variation in sperm function (Sofikitis et al. 1995; Jensen et al. 2004; Eskenazi et al. 2005; Aitken et al. 2014). Several environmental factors have also been shown to affect sperm function in livestock (Dziuk 1996) and in insects, including sexually transmitted microbes (Otti et al. 2013) or the storage environment in the female (Ribou and Reinhardt 2012), and this altered sperm phenotype impacts on reproductive success (Dziuk 1996; Reinhardt and Ribou 2013). Environmental effects can also accumulate over time, a process known as sperm aging whereby longer exposure to the environment usually causes stronger effects (Reinhardt 2007; Pizzari et al. 2008a). Sperm function after a period of sperm aging is, therefore, an outcome of the interaction between male genotypic and environmental effects. The accumulation of environmental damage in sperm can even lead to transgenerational fitness effects, such as increased cancer incidence in children fathered by smokers (Aitken et al. 2014), imprinting alterations in offspring sperm due to parental fungicide exposure (Stouder and Paoloni-Giacobino 2010), or a range of health problems and diseases when embryos arise from fertilization with aged sperm (Tarín 2000; Reinhardt 2007; Pizzari et al. 2008b; Tan et al. 2013). Therefore, selection is expected to operate on males and females to reduce the access of environmentally, sublethally damaged sperm to fertilization (Reinhardt 2007). While the female immune system has been specifically suggested to affect cryptic female choice (Eberhard 1996), immune and proteome responses can also be directed against damaged or aged sperm (Liljedal et al. 1999; Georgiou et al. 2005; Peng et al. 2005; Naz 2006, Radhakrishnan and Fedorka 2012). If such response included damaged or aged sperm of the same genotype (Reinhardt and Siva-Jothy 2005; Tan et al. 2013), it would not represent cryptic female choice.

To summarize, despite the identification of a suitable candidate to affect sperm function (the female immune and proteome response) and despite its fundamental importance for reproductive medicine and evolutionary biology, the relative significance of environmental and genetic variation in sperm function has rarely been tested, if it had, as in some *in vitro* studies on artificial insemination (see Dziuk 1996), dramatic environmental effects were found. Here we compare gene expression in females that received sperm from 1 of 3 different inbred male populations (genotypes) from either of 2 environmental treatments. The environmental treatments consist of either short or long exposure of ejaculate components in the storage organ of same-aged males. For brevity, we denote them as sperm storage times, being aware that, akin to sperm competition, "sperm" here includes additional ejaculate components. We use bedbugs, *Cimex lectularius*, an insect that has several important properties as a model system for our question: 1) After mating, sperm move freely through the female body, and hence are directly exposed to the female proteome and the immune system. 2) Long exposure in females reduces the fertilization ability of sperm (Mellanby 1939) and leads to female infertility (Reinhardt and Ribou 2013) showing that negative fitness effects can arise when sperm in storage are exposed to the female environment, and selection against environmental damage may be expected. 3) Female immune responses in bedbugs were specifically suggested to be directed toward certain sperm genotypes and so cause evolution by cryptic female choice (Eberhard 1996). Finally, 4) premating female choice is unlikely because the mating rate of bedbug females is controlled by males (Reinhardt et al. 2009)—any female choice that occurs will be largely based on ejaculate characteristics. Although postcopulatory sexual selection predicts that females respond to sperm genotypes (e.g., Eberhard 1996; Lüpold et al. 2013), previous studies have, to our knowledge, not accounted for the possibility that different male genotypes respond differently to the same environment, and that females may actually respond to the environmental rather than the genetic component of the sperm phenotype. Here we examine whether such female responses toward the environmental component of the sperm phenotype are possible at all. To obtain a quantitative measure of this effect we compare the female gene expression response to the environmental and the genotypic component of sperm. In order to have a simple design in this proof-of-concept experiment we used females from only one population. Although this approach avoided complex genotype × genotype interactions, it still allowed us to assess the possible effect of female responses to coevolved (same population) or noncoevolved sperm genotypes. By examining the environmental effect within different sperm genotypes, we were also able to investigate the presence of any environment × sperm genotype effects.

MATERIALS AND METHODS

Bedbug culture and reproductive biology

All bedbugs (origin see below) were maintained in an insectary at 26 ± 1 °C, at 70% relative humidity with a cycle of 12L:12D. The feeding and maintenance protocol follows Reinhardt et al. (2003). All individuals in our study were virgins to begin with and were kept individually in 15-mL bijou tubes provided with a piece of filter paper.

Bedbugs mate by traumatic insemination, that is, the male injects sperm through the punctured female abdominal body wall into a special organ, the mesospermalege, in the female body cavity. Between 1 and 4 h after mating sperm migrate from the mesospermalege into the hemolymph and reach the oviduct after around 4–8 h from where they either are stored in specialized organs or move through the oviduct walls to fertilize the eggs within the ovary (reviewed in Usinger 1966; Reinhardt and Siva-Jothy 2007).

Sperm phenotype

A phenotype is determined by its genotype and the environment. The same applies to the sperm phenotype (Parker and Pizzari 2010), which is here partitioned into a male genotypic and an environmental component. The environmental component combines effects on sperm from the time of production to the actual fertilization of an egg, including environmental effects on male physiology that translate into effects on sperm function. To facilitate reading, in the Methods and the Results section, we refer to the environmental component of sperm and the ejaculate simply as E, and to the male genetic component of sperm as G and its interaction as G × E.

Sperm genotype (G)

We used males from 3 large stock populations (>1000 individuals) of different origins, called *A*, *B*, and *C* to represent genotypes. The great differentiation between, and very low differentiation within,

natural populations of bedbugs (Fountain et al. 2014) is mimicked in the laboratory and likely represents a simple surrogate for genotype. Although not necessary for genetic differentiation (Fountain et al. 2014), we point out that the populations also have very different collection origins. *A* is of unknown origin in the wild but has been maintained at the University of Sheffield for >10 years and before that for >40 years at the London School of Hygiene and Tropical Medicine, *B* was collected in London, and *C* in Nairobi, 4 and 2 years prior to the experiment, respectively. Final-instar nymphs from the 3 populations were isolated to produce virgin individuals. Males were collected upon eclosion and randomly assigned to 2 different E treatments (see below).

Environment of sperm (E)

We used 2 very simple environments: We altered the period between sperm production and mating thereby creating 2 sperm cohorts that were exposed to the same environment (storage organ in the male in our laboratory) but for different periods (i.e., different times over which damage could accumulate; see details below). In this way, we were able to keep 4 important parameters constant: the male age at mating, as well as sperm volume, seminal fluid volume, and the proportion of live sperm cells per ejaculate at the time of mating (Supplementary Data). We were not able to simultaneously also keep constant the male age at which the sperm is produced. Nevertheless, this protocol manipulated a nongenetic, that is, E, component of the male and henceforth we refer to short and long sperm storage to refer to our sperm storage, or E, treatment.

All males were fed twice prior to mating. Males of the long storage treatment were fed 5 and 4 weeks prior to mating, creating high early and very low late sperm production because early produced sperm were exposed to the environment for a long time while being "diluted" with few, or no, recently produced sperm (Supplementary Data). Males of the short storage treatment were fed at 2 weeks and 1 week prior to mating, creating very little early and high late sperm production because most sperm will have been produced just prior to mating and exposed only briefly to the environment (Supplementary Data). Therefore, the relative amount of aged sperm is assumed to be much higher in the long than in the short sperm storage treatment.

Experimental design

Ninety-six virgin females from the population *A* were mated to a male from either of the 3 populations (see Materials and Methods above) (G). These males either had short- or long-storage sperm phenotypes, amounting to 6 groups of 16 females each. Two females were pooled in order to provide sufficient RNA (see RNA extraction, purification, and complementary DNA [cDNA] synthesis below) resulting in 8 replicates per treatment. Females were mated for 60 s to adhere to previously described protocols (Reinhardt et al. 2003; Reinhardt et al. 2009) and to reduce variation in sperm number (Siva-Jothy and Stutt 2003).

Mated females were randomly assigned to either of 2 groups in order to sample a greater time window of the important postcopulatory process of the sperm traveling to the ovaries. The first group was sampled 1 h after mating (room temperature), representing any initial gene expression when all the sperm are in the female immune organ (Usinger 1966). The second group was left at room temperature for 6 h representing gene expression when sperm pass through the hemolymph (sperm leave the immune organ [mesospermalege] after 2–4 h—Usinger 1966). At the prescribed times, the abdomen of a female was separated from head and thorax and immediately immersed in RNAlater (Qiagen, Manchester, UK). For the differential gene expression analysis the whole postcopulatory response was combined in order to gain replicates, that is, grouping females incubated for 1 and 6 h after mating respectively (there were very small effects of time—Supplementary Data). To exclude that any of the observed effects were due to mating per se as shown for *Drosophila* (Chapman et al. 2003; Lawniczak and Begun 2004; McGraw et al. 2004; Mack et al. 2006) and *Anopheles* (Dottorini et al. 2007) and to have a baseline gene expression level we compared gene expression in 8 unmated females after 1 h and 8 unmated females after 6 h incubation at room temperature. These females were also handled in the same way as the mated females except for the mating and any male contact. All experimental steps up to the RNA extraction were completed on the same day.

RNA extraction, purification, and cDNA synthesis

To obtain sufficient material, we had to pool 2 RNAlater-stabilized female abdomens from the same treatment group. RNA extraction and purification were carried out using the RNAeasy Kit (Qiagen). Tissue was homogenized for 10 min using buffer and a tissue homogenizer (TissueLyser; Qiagen). Total RNA from 56 tissue samples was eluted in RNase-free water and quantified using a NanoDrop 8000 (Thermo Scientific, Cramlington, UK) and Agilent 2100 Bioanalyzer (Agilent, Winnersh, UK). The RNA concentrations for each sample were standardized to 150 ng/μL for the cDNA synthesis. We then pooled all samples from one treatment per genotype group to standardize for individual variation in expression ($N = 14$ treatment groups). From the 14 samples double-stranded cDNA was constructed using the Evrogen MINT cDNA synthesis kit according to the manufacturer's protocol and then purified using a QIAquick PCR purification kit (Qiagen). The construction of barcoded 454 GS FLX titanium sequencing libraries and sequencing on 2 picotiter plates were carried out by the Centre for Genomic Research (Liverpool, UK).

Sequence assembly and annotation

Raw reads were trimmed and assembled using gsAssembler from Newbler 2.6. Assembled contigs and isotigs were annotated with BLAST results and gene ontology (GO) terms using BLAST2GO 2.5. Sequences were first searched against the NCBI nonredundant (nr) protein database using BLASTx with an *e* value cutoff of 10^{-3}. Sequences that retrieved no hits were then searched against the NCBI nr nucleotide database using BLASTn with an *e* value cutoff of 10^{-10}. GO terms were extracted from BLAST results using GOSlim in BLAST2GO as described elsewhere (Conesa et al. 2005). Pyrosequencing yielded 1 158 181 high-quality reads which were assembled into 13 045 isotigs of mean length 839 bp and N50 1180 bp; 73% of isotigs retrieved a blastx hit (*e* value $<10^{-3}$).

Differential gene expression analysis

To create a reference for read mapping, the longest isoform of each contig or isotig was extracted from the gsMapper 454Isotigs.fna assembly file. Trimmed reads from each treatment were mapped to the reference using gsMapper from Newbler 2.6 and differential gene expression was determined using the R package *DESeq2*, which uses the negative binomial distribution and a shrinkage estimator to determine variance–mean dependence in mapped read counts and a conditional test for differential expression (Love et al. 2014). The cut-off value for significant differential expression was set to $P < 0.05$, after adjustment using false discovery rate. We

normalized the EST library using the estimateSizeFactors function implemented in *DESeq2* and identified differential expression between treatment groups using the DESeq function by fitting within the mated samples the 2 factors G (sperm genotype), E (sperm environment) and its interaction, G × E on female gene expression. From this analysis we extracted the log2 fold changes for specific contrasts, that is, short-stored versus long-stored sperm as well as the pairwise differences between sperm genotypes in female gene expression between G (*B* vs. *A*, *C* vs. *A*, and *C* vs. *B*). Further, we extracted the log2 fold changes for the interaction term to look at the sperm genotype-by-environment interaction. To present the sperm genotype-by-environment interaction in a more comprehensible way we give both E effects within each G (Table 2).

For the baseline gene expression level, in a second model we compared gene expression differences between mated and virgin samples. For methodological reasons we had to accept a shortcoming of comparing 2 virgin to 12 mated samples is statistically limited—if there is high variance in expression between the virgin samples, the probability of finding differentially expressed genes may be reduced.

RESULTS

Female gene expression in response to E

Seventy-nine genes were differentially expressed in response to E. Twenty-one out of these were expressed significantly higher in the long sperm storage treatment (Table 1 and Supplementary Table S3), 58 significantly lower compared with the short sperm storage treatment (Table 1 and Supplementary Table S3). For a nonmodel organism, our transcriptomic study returned a relatively high proportion of predicted proteins (ca., 72%) among the expressed sequences. Of the genes differentially expressed in response to E, 45 of the 79 coded for various proteins including cuticular, cell cycle, metabolic, and mitochondrial proteins (Supplementary Table S3), whereas 34 genes were unannotated transcripts. The average response magnitude was 0.90 ± 0.32, with 20 genes showing expression levels log2 fold change of >1.0 (Figure 1).

Female gene expression in response to G

In total 4 genes were differentially expressed in females in response to the sperm genotype (Table 1 and Supplementary Table S2). Two genes were expressed significantly lower in females exposed to *B* sperm compared with *A* sperm. Two genes were expressed significantly higher in females exposed to *C* sperm compared with *B* sperm, one of them also differed in response to *A* sperm (CL-00937) (Table 1 and Supplementary Table S2). The same gene (CL-00937) was as well significantly differentially expressed in response to E, although at a smaller magnitude (Supplementary Figure S3). The average response magnitude was 1.52 ± 0.55, all genes showed expression levels log2 fold change of >1.0 (Figure 1).

Female gene expression showing G × E interaction effects

Fitting G × E interactions on female gene expression revealed different gene expression of 8 genes, 1 in response to coevolved G (i.e., *A* sperm) and 7 with noncoevolved G (*B* or *C* sperm) (Table 2). The average response magnitude was 2.03 ± 0.94, all genes showed expression levels log2 fold change of >1.0 (Figure 1).

Specifically, females showed higher expression of 4 genes in response to short- than long-stored *B* sperm, 3 of them with unannotated transcripts. In response to *C* sperm, a lysozyme gene and 2 sequences with no similarity were higher expressed after exposure to long- than short-stored sperm (Table 2). Female response to long-stored sperm differed with G (Supplementary Table S4): relative to long-stored *A* sperm, 1 gene was higher and 2 were lower expressed in response to long-stored *B* sperm, whereas 1 lysozyme gene was higher expressed in response to long-stored *C* sperm (Supplementary Table S4). After exposure to long-stored *C* sperm, females expressed 5 genes significantly higher compared with long-stored *B* sperm, 4 of those sequences were unannotated transcripts. In response to long-stored *B* sperm 1 gene (CL-05792: no similarity) was significantly lower expressed relative to long-stored *A* and *C* sperm (Supplementary Table S4).

The effect of mating per se

To put our results in perspective, we compared gene expression of mated females to virgins to detect the effect of mating per se. Sixteen genes were significantly differently expressed between mated and virgin females (Table 1 and Supplementary Table S1). Six genes showed significantly lower, and 10 genes significantly higher, expression levels in mated than virgin females (Table 1 and Supplementary Table S1). Thirteen of the significantly differently expressed genes were annotated to a known gene sequence and 3 were genes of unknown function (Supplementary Table S1). The average response magnitude was 1.37 ± 0.40, 13 genes showed expression levels log2 fold change of >1.0 (Figure 1). Four of the genes (CL-01375, CL-02863, CL-05537, CL-06015) that responded to mating per se were also differentially expressed in response to E, all positive (i.e., upregulated), two >1.0 and two <1.0 log2 fold change (Supplementary Figure S3).

DISCUSSION

In an important model species of sexual selection we found strong support for the idea that females respond to the environmental component of the sperm phenotype. By contrast, the female

Table 1

Number of significant female gene expression differences in response to mating, sperm genotypes, and sperm environment (short vs. long storage)

Direction of gene expression difference	Mated versus virgin	Response to genotypes			Short versus long sperm storage
		B versus *A*	*C* versus *A*	*C* versus *B*	
Higher	10	0	1	2	58
Lower	6	2	0	0	21

The direction of expression difference refers to higher versus lower expression. "Higher" and "lower" refers to the group indicated first in the comparison.

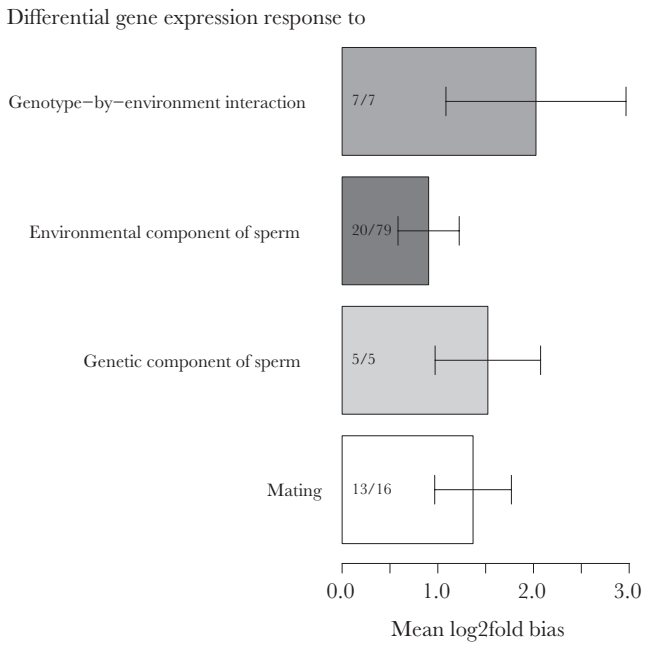

Figure 1
Mean magnitude of differential gene expression response (mean log2 fold bias) in females for each factor and the genotype-by-environment interaction. In each bar the number of genes with a log2 fold change >1 and the total number of significantly differentially expressed genes found for each factor and the interaction term are indicated. Error bars represent one standard deviation.

response to the genotypic component was much weaker in terms of the number of genes involved. This finding suggests that a female response to variation in the sperm environment exists (equivalent to postcopulatory natural selection). To the extent that the average effect per gene is the same across treatments, it also suggests that it can be stronger than postcopulatory sexual selection. These comparisons may even be conservative given that the genetic variation between males within a population (the one sexual selection can act on) may be smaller than the genotypic differences between populations (Fountain et al. 2014) that we employed. By contrast, the environmental manipulation we used—sperm storage variation introduced by food suppression—is unlikely to be unusual in nature.

We also provide rare evidence that any response to sperm genotypes depends on the sperm environment. Such genotype-by-environment interaction effects at the sperm level are hitherto rarely documented (Axelsson et al. 2010) and even at the premating level have only recently come into focus (reviewed by Ingleby et al. 2013). Finally, our study confirms findings in other organisms that females alter gene expression purely in response to mating (Chapman et al. 2003; Lawniczak and Begun 2004; McGraw et al. 2004; Mack et al. 2006; Dottorini et al. 2007). Below we discuss some of the consequences of these findings for several areas in biology.

Sperm environmental and genotypic components

In an attempt to assess the relative contribution of heritable and nonheritable sperm effects on females under natural conditions, we found almost 20 times more genes are differentially expressed in response to nonheritable sperm variation (E) than to heritable sperm variation (G). Particularly when considering the sensitivity of our analysis, reflected by the effect of mating per se (5 times as many genes) the effect constitutes a substantial female response to nonheritable sperm characteristics. Because of our design, there were 4 samples per male genotype in the G, but 6 for each E treatment. This may for statistical reasons render more genes significant in the E treatment. However, if we confine the E-G comparison to genes with a log2 fold change >1, there were still 20 genes in the E compared with 4 genes in the G treatment. We, therefore, suggest that our finding of a strong female response to E is not a statistical artifact.

That females so substantially alter gene expression in relation to the sperm environment (Supplementary Table S3) suggests that females are selected to circumvent environmental effects that damage sperm. By expressing genes that initiate the removal of exposed, damaged, or aged sperm components, females may be able to increase the amount of fertile eggs even when they receive substantial amounts of such sperm. A direct test of this hypothesis would require measuring female fitness in the absence of the expression of these specific genes. Even though this is likely to pose an experimental challenge in animals not amenable to silence specific genes by RNA interference, the genes we identified may be a fruitful first step to identify candidate genes.

Under the hypothesis that older sperm, which were longer exposed to the male environment, have negative effects on females, females are expected to benefit from stronger expression of genes linked to the removal of aged, dysfunctional sperm (Reinhardt 2007; Pizzari et al. 2008a). Alternatively, sperm may have evolved delayed activation, such as after a certain time in storage (Dziuk 1996), in which younger sperm would have reduced functionality and females may benefit from removing them. Our study did not test this idea directly but we found that females had higher expression in almost three-quarters of the differentially expressed genes in response to short-stored sperm relative to long-stored sperm (Supplementary Table S3). This suggests that females may be more responsive to fresh sperm and ejaculate components.

As we noted in the introduction, "sperm" includes sperm cells and other components, which may change with storage time. Seminal substances affect female physiological responses (Avila et al. 2011; Perry et al. 2013) and, therefore, gene expression. Whether or not, and how much and how quickly the chemical nature, or composition, of the seminal fluid changed over the sperm storage duration investigated here is not currently known for insects. It would, however, still constitute an environmental, not a genotypic alteration. If during storage some seminal fluid components lost functionality to which females usually respond, it may explain our observation that females showed a weaker response toward ejaculates from the long sperm storage treatment. Future studies should test this idea directly. Similarly, the feeding regime, that is, the time between feeding and ejaculation, might have had an effect on sperm. Our protocol did not allow us to separate the effect of time between feeding and ejaculation from the time of sperm production to ejaculation. However, both parameters would exert environmental, or G × E effects. Certainly, our feeding protocol was not imposing anything unusual given that bedbugs can live for a year and food availability is unlikely to be predictable (Usinger 1966).

The female response to the nongenetic component of sperm suggests the environmental component of sperm may be an important predictor of reproductive variation. Studies in sexual selection may, therefore, benefit from taking into account nonheritable

Table 2

Female gene expression in response to sperm phenotypes illustrating the female response to sperm genotype by environment interactions

Gene ID	Annotation	GO[a]	InterPro signature	Log2 fold change[b]	Adjusted *P* value[c]
Stock population A					
CL-11639	A-kinase anchor protein	F: RNA binding	IPR002999; IPR004087; IPR004088; IPR006021	1.758	0.029
Stock population B					
CL-03617	Apolipoprotein D-like precursor	F: pigment binding; lipid binding; transporter activity; receptor activity; small molecule binding; cholesterol binding P: transport; evasion or tolerance of host defense response; cell differentiation; nervous system development; multicellular organismal development; brain development; negative regulation of smooth muscle cell proliferation; response to drug; negative regulation of lipoprotein lipid oxidation; tissue regeneration; response to axon injury; negative regulation of smooth muscle cell–matrix adhesion; negative regulation of protein import into nucleus; negative regulation of platelet-derived growth factor receptor signaling pathway; negative regulation of T-cell migration; peripheral nervous system axon regeneration; negative regulation of focal adhesion assembly; response to reactive oxygen species; negative regulation of monocyte chemotactic protein-1 production; aging; lipid metabolic process; negative regulation of cytokine production involved in inflammatory response; glucose metabolic process C: region; membrane; anchored to membrane; plasma membrane; perinuclear region of cytoplasm; endoplasmic reticulum; extracellular space; cytosolic ribosome; neuronal cell body; dendrite	IPR000566; IPR011038; IPR012674	1.887	0.016
CL-04603	No similarity	NA		1.941	0.011
CL-05792	No similarity	NA		3.949	0.001
CL-05809	No similarity	NA		1.159	0.012
Stock population C					
CL-04459	Lysozyme	F: lysozyme activity; hydrolase activity; hydrolase activity, acting on glycosyl bonds; catalytic activity P: cell wall macromolecule catabolic process; metabolic process; cytolysis; defense response to bacterium	IPR001916; IPR019799; IPR023346	−1.748	0.043
CL-05678	No similarity	NA		−1.008	0.043
CL-05792	No similarity	NA		−2.758	0.043

The fold change compares gene expression levels of females exposed to short-stored relative to females exposed to long-stored sperm. Each sperm genotype is presented individually to give an indication of the combined effect of sperm genotype and sperm environment. The numbers assigned to the unknown genes were randomly chosen for the purpose of contig identification.

[a]GO: C, cellular component; F, molecular function; P, biological process.

[b]Log2 fold changes are relative to long-stored sperm.

[c]Used adjusted *P* value cutoff of 0.05.

components of the sperm phenotype. As argued previously (Reinhardt 2007), male or female traits that alter the environmental components of sperm, or the number of sperm with certain environmental signatures, may be under natural or sexual selection. This brings about the interesting situation that postcopulatory variation in sperm traits is environmental but may nevertheless be inherited if male or female components affect the sperm environment. As a large number of studies show time-related and other environmental variation in the sperm phenotype (e.g., Poland et al. 2011; Ribou and Reinhardt 2012; Otti et al. 2013) male or female traits influencing the sperm environment may be widespread.

Concluding remarks

Longer precopulatory male sperm storage resulted in higher expression of some genes in females, including stress response genes. That the sperm genotype induced the expression of roughly 20 times fewer genes than the sperm environment significantly advances our understanding of variation in reproductive success. Incorporating the nongenetic component of sperm into experimental designs of sexual selection studies is likely to increase the power of predicting reproductive success. It will be more difficult to control for the fact that different sperm genotypes respond differently to the same

environment (genotype-by-environment interaction—Axelsson et al. 2010). More generally, our results suggest that natural selection in different sperm environments may play a major role in the evolution and diversification of reproductive traits, in addition to sexual selection.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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