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Cryptic haplotype-specific gamete selection yields offspring with optimal MHC immune genes

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Females choose specific mates in order to produce fitter offspring. However, several factors interfere with females' control over fertilization of their eggs, including sneaker males and phenotypically unpredictable allele segregation during meiosis. Mate choice at the individual level thus provides only a poor approximation for obtaining the best genetic match. Consequently, postcopulatory sperm selection by female oocytes has been proposed as a mechanism to achieve complementary combinations of parental haplotypes. Here, using controlled in vitro fertilization of three-spined stickleback eggs, we find haplotype-specific fertilization bias toward gametes with complementary major histocompatibility complex (MHC) immunogenes. The resulting zygote (and thus offspring) genotypes exhibit an intermediate level of individual MHC diversity that was previously shown to confer highest pathogen resistance. Our finding of haplotype-specific gamete selection thus represents an intriguing mechanism for fine-tuned optimization of the offspring's immune gene composition and an evolutionary advantage in the Red Queen dynamics of host-parasite coevolution.

KEY WORDS: ***Gasterosteus aculeatus*, in vitro fertilization, major histocompatibility complex, MHC divergence, postcopulatory sexual selection, three-spined stickleback.**

Cryptic female choice describes a suite of postcopulatory mechanisms allowing a female control over which male's sperm ultimately fertilizes her eggs in promiscuous mating systems. These mechanisms are thought to aim at both inbreeding avoidance and increasing the female's fitness through preferential fertilization by a male with a heritable advantageous trait, that is the acquisition of good genes (Eberhard 1996; Birkhead and Pizzari 2002). However, for some traits there is no single good gene and optimal offspring genotypes result from a complementary combination of female and male haplotypes (Brown 1997; Kempenaers 2007; Firman et al. 2017). One of the best-studied examples for complementary, rather than absolute, genetic quality is the highly polymorphic major histocompatibility complex (MHC) (Milinski 2006). As part of the adaptive immune system of vertebrates, MHC molecules present antigens at the cell surface and thus play a key role in the recognition of invading pathogens. Precopulatory mate choice for males with complementary whole MHC genotypes presumably evolved as a step toward optimizing the overall immune response in the offspring (Edwards and Hedrick 1998; Ziegler et al. 2005; Milinski 2006). Yet, if sexual selection targets a particular locus or genomic region, precopulatory selection allows only for a poor approximation of the best match. Ultimately, the combination of a maternal and a paternal haplotype, resulting from phenotypically unpredictable meiotic segregation among both oocytes and sperm, determines the offspring's genotype. In highly heterozygous systems, such as the MHC, random fusion of gametes to some extent resembles a lottery and can easily lead to nonoptimal haplotype combinations.

Imagine for instance a female with the two homologous haplotypes A and B, and a male with the two haplotypes B and C. Now say that A would be most complementary to B, and B most complementary to C. At the individual level, these mates seem like a perfect match. After random meiotic segregation though, half of the resulting zygotes will have either BB or AC genotypes, both of which are noncomplementary, leaving half of the offspring with suboptimal genotypes despite costly investment in precopulatory mate choice. Eggs that distinguish between sperm haplotypes could provide a solution to this dilemma, allowing for the combination of the most compatible parental haplotypes. The ability of eggs to prefer complementary sperm haplotypes would thus provide a great evolutionary advantage even in nonpromiscuous mating systems (Eberhard 1996; Bernasconi et al. 2004; Otto et al. 2015), irrespective of whether it evolved before or after precopulatory mechanisms. However, it is interesting to note that postcopulatory mechanisms of sexual selection are generally predicted to evolve earlier than precopulatory mechanisms, suggesting that MHC-based gamete selection might predate MHC-dependent mate choice and might be operating even in species where no MHC-dependent mate choice has been observed thus far.

The MHC has been proposed as one of the prime candidates for a target of complementary cryptic female choice (Wedekind 1994; Birkhead and Pizzari 2002; Ziegler et al. 2005; Firman et al. 2017). At the individual (diploid genotype) level, sperm selection could indeed already be detected in several species, interestingly targeting different extremes of MHC diversity. In Atlantic salmons, where eggs in vitro were given the choice between sperm from MHC-identical and from completely MHC-dissimilar males, sperm from MHC-identical males was more successful in fertilization (Yeates et al. 2009). Similar observations of a postcopulatory fertilization advantage for MHC-similar mates were also made in Chinook salmon (Geßner et al. 2017), guppies (Gasparini et al. 2015), salamanders (Bos et al. 2009), and kestrels (Alcaide et al. 2012). Conversely, in red junglefowl, fertilization of eggs was biased toward sperm from MHC-dissimilar males (Løvlie et al. 2013). Another hint comes from crossing congenic laboratory strains of mice that supposedly differed only at the MHC. These studies suggest nonrandom combinations of parental MHC haplotypes in the resulting blastocysts. However, the pattern was not consistent and was affected by parental infection levels with mouse hepatitis virus (Wedekind et al. 1996; Rülicke et al. 1998). Segregation of MHC haplotypes in mice may also be distorted by other mechanisms, such as the *t-haplotype complex* that induces meiotic drive and happens to overlap with the MHC locus (Lyon 2003). Furthermore, while a study on Atlantic salmon showed MHC-dependent cryptic mate selection at the individual level in matings with multiple males (Yeates et al. 2009), investigations of cryptic choice at the gamete level in single-male matings of salmon as well as whitefish revealed only random fertilization with regard to MHC (Wedekind et al. 2004; Promerova et al. 2017). Thus, decisive experimental evidence for oocyte selection of specific sperm haplotypes is still elusive (Firman et al. 2017).

Using the externally fertilizing teleost model species three-spined stickleback (*Gasterosteus aculeatus*) (Gibson 2005) we test here whether fertilization of eggs by sperm is nonrandom with regard to the MHC IIB haplotype combinations of the two gametes. Such sexual selection at the gamete level could be expected to evolve as an extension of the well-characterized precopulatory mate choice for MHC compatibility (Milinski et al. 2005), potentially compensating for uncontrollable factors such as the random meiotic haplotype segregation during egg and sperm development. MHC haplotypes are defined as extended chromosomal stretches with high linkage disequilibrium that contain the MHC genes, but also a substantial number of other genes in their vicinity, including olfactory receptor genes suggested to be involved in sexual selection (Horton et al. 2008; Ziegler et al. 2010). In order to investigate MHC-specific fertilization effects at the gamete level, we therefore focused our main analysis on egg-sperm combinations that differed in their MHC haplotypes. This is particularly important for the interpretation of any observed MHC effect, as a mere dichotomous distinction of MHC identical/different haplotypes, used in previous studies, applies equally to all other non-MHC genes in the chromosomal vicinity of those haplotypes: Any observed fertilization advantage (or disadvantage) for egg-sperm combinations with identical MHC haplotypes could thus not be reliably attributed to the MHC alone (Ziegler et al. 2010). To quantify the similarity/dissimilarity between different parental MHC haplotypes, we used the average sequence divergence between the MHC IIB alleles on those haplotypes. Sequence divergence between MHC alleles has become a standard proxy for estimating the functional quantitative difference in antigen-binding between the respective MHC proteins and is extremely unlikely to correlate with similarity at any other gene in the vicinity of the MHC loci (Forsberg et al. 2007; Lenz 2011; Evans et al. 2012; Geßner et al. 2017).

MHC IIB loci in three-spined sticklebacks occur in a variable number of tightly linked gene copies and are thus inherited in stable haplotype blocks with one to three alleles each (Lenz et al. 2009a; Eizaguirre et al. 2012). As a measure of genetic divergence between the MHC IIB haplotypes of a female's egg and a male's sperm, we therefore used the average pairwise amino acid-based sequence divergence between all parental MHC IIB alleles on those two haplotypes and refer to this measure as "egg-to-sperm MHC divergence" throughout. The eggs of a female stickleback were exposed, in vitro, to equal volumes of sperm from two males simultaneously, resulting in the availability of four male MHC IIB haplotypes for fertilization of each egg-haplotype (Fig. 1), each of

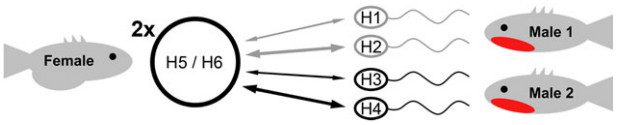

Figure 1. Design of in vitro fertilization experiment for haplotype-specific gamete selection.
Experimental design: Female eggs were presented with sperm from two different males under controlled conditions in vitro. Each gamete (sperm or egg) carries one of two randomly segregating parental MHC IIB haplotypes (e.g., H1). Arrows depict specific combinations of egg and sperm with a distinct egg-to-sperm MHC divergence (e.g., divergence between H1 and H5). Initial analyses are based on all observed combinations of parental MHC haplotypes after in vitro fertilization (black and gray arrows). To exclude interindividual differences between males, downstream analyses concentrate on relative fertilization success of the two sperm haplotypes within each male (black arrows only), with one of them being randomly selected as focal haplotype. Pictured haplotypes (H1–H6) are only examples and not meant to depict the actual MHC diversity in the experiment.

them representing a distinct egg-to-sperm MHC divergence. This scenario with simultaneous fertilization by two males, mimicking the common situation of a sneaking neighbor male fertilizing the eggs almost simultaneously, was chosen to guarantee sufficient variation among the sperm available for each egg's fertilization and thus increase the likelihood to observe cryptic gamete selection, but also to allow replication of previous reports of postcopulatory MHC-based mate selection.

Results

Our initial dataset comprised 11 clutches for which a total of 890 developing eggs could successfully be genotyped for their MHC IIB haplotype combinations. A median of 83 (±11 SD) eggs per clutch allowed us to reliably estimate fertilization success of the different male and female MHC haplotype combinations. First we investigated whether fertilization was nonrandom with regard to MHC. For this, a Monte Carlo sampling procedure was employed to simulate random fertilization of the four sperm MHC haplotypes (i.e. ~25% each), originating from the two males available to each egg with a given MHC haplotype. Random sampling was based on the actual observed number of fertilized eggs of each of the females and their MHC haplotypes, for each egg randomly picking one of the four specific sperm MHC haplotypes available to that given egg. This analysis revealed that the observed average deviation from 25% in a sperm haplotype's fertilization success was significantly larger than expected under random fertilization ($P < 0.001$; Fig. S1). We then explored whether a directional preference for the sequence divergence between egg and sperm could be identified. To test whether fertilization was skewed toward higher or lower egg-to-sperm MHC divergence, we compared the observed mean egg-to-sperm divergence to a distribution of egg-to-sperm divergence obtained from simulating random mating. However, this analysis revealed no preferential fertilization by sperm haplotypes yielding low or high egg-to-sperm MHC divergence (Mean observed: 0.2390, mean expected: 0.2398, $P = 0.91$; Fig. 2A), so no trend for minimizing or maximizing MHC diversity was found. The observed intermediate mean MHC divergence in the fertilized zygotes could imply either random gamete fusion or an active preference for intermediate MHC diversity. To distinguish between these two scenarios, we compared the variance in egg-to-sperm MHC divergence among the observed zygotes against the variance resulting from random fertilization, again using a Monte Carlo sampling procedure. In contrast to a recent study in Atlantic salmon, we indeed found that the observed fertilization events led to zygotes with a lower variance in egg-to-sperm MHC divergence than zygotes formed by randomly sampled sperm haplotypes (variance observed: 0.00082, variance under random fertilization: 0.00166, $P = 0.015$; Fig. 2B). This observed pattern could result from sperm selection by eggs, focusing on specific male haplotypes that yielded intermediate egg-to-sperm MHC divergence. Indeed, when ranking the four sperm MHC haplotypes available to a given female egg according to their fertilization success and comparing the most successful sperm haplotypes with the least successful ones, we found that the former were closer to the mean MHC divergence of the population, possibly approximating the optimal MHC divergence in an individual (Wilcoxon signed-rank test, $P = 0.0018$; Fig. 2C).

Encouraged by these results, we then moved forward with our main analysis. Here, we used generalized linear-mixed models (GLMMs) to investigate a potential optimal egg-to-sperm MHC divergence without making assumptions about the exact location of this optimum. Furthermore, this model-based approach allowed us to account for differences in sperm concentration among males, a parameter likely affecting fertilization success. For model-based analyses, we used only a high-quality subset of the data. The filtering criteria for this subset aimed to avoid pseudo-replication through repeated counting of identical haplotypes in homozygous individuals and excluded gamete combinations with identical MHC haplotypes for reasons explained above (see also method section and Fig. S2). The model-based analyses confirmed the initial results: We found a quadratic association between egg-to-sperm MHC divergence and the proportion of fertilized eggs ($N = 73$; GLMM, $X^2_{1,67} = 12.6$, $P = 0.0004$; Table 1), suggesting that sperm resulting in a more intermediate egg-to-sperm MHC divergence were more successful in fertilizing the respective eggs. This association was independent of a similarly strong effect of sperm number on fertilization success (GLMM, $X^2_{1,67} = 12.7$, $P = 0.0004$). We also tested whether the removal of

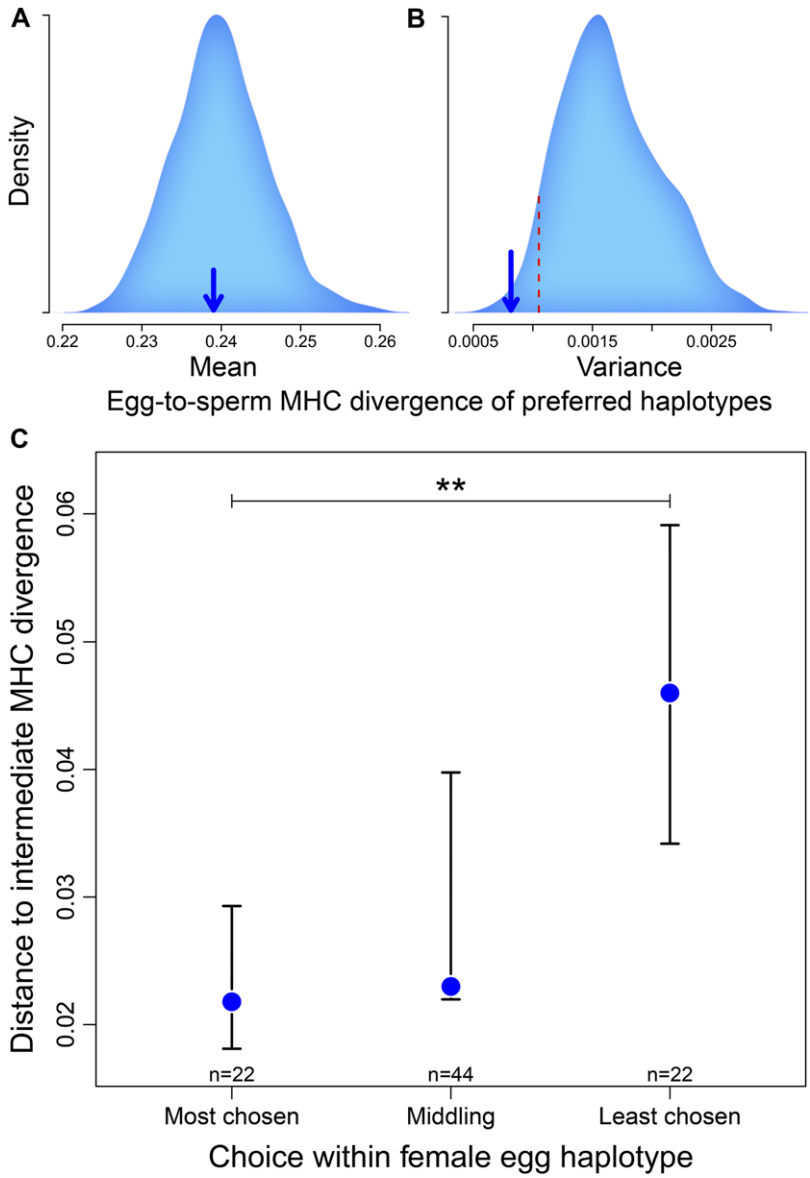

Figure 2. **Nonrandom fertilization success with regard to MHC immune genes.**
(A) Comparison of observed average egg-to-sperm MHC divergence (arrow) to simulated random fertilization by available sperm haplotypes (shaded area, 1000 simulation runs) revealed no preference for maximum or minimum MHC divergence, indicating either truly random fertilization or preference for intermediate MHC divergence. (B) Observed fertilizations (arrow) exhibited lower variation in egg-to-sperm MHC divergence than expected under random fertilization (shaded area, 1000 simulation runs), suggesting a preference for intermediate MHC divergence. Dashed red line indicates 5% quantile of the distribution. (C) The deviation (median absolute distance ± 95% CI) between a sperm's egg-to-sperm MHC divergence and a hypothetical population optimum (0.2356) is shown here for the most successful ("Most chosen"), the least successful ("Least chosen") and the two middling of the four sperm haplotypes available for fertilizing a given egg haplotype ($N = 44$, see Fig. 1). ** = $P < 0.01$.

gamete combinations with identical MHC haplotype (one of our quality filtering criteria) affected the results and only found that their inclusion slightly reduced the significance of the quadratic association ($N = 79$; GLMM, $X^2_{1,73} = 11.9$, $P = 0.0006$). This is in line with our concern that fusion of gametes with identical haplotypes might follow a different dynamic than the one we are interested in here (see arguments in introduction and discussion). However, while mimicking promiscuous mating, this analysis still included comparisons of sperm from different males, where the influence of MHC-independent traits other than sperm number (e.g., sperm velocity or seminal fluid; Perry et al. 2013) is not controlled for. In principle, such MHC-independent traits should affect both MHC haplotypes of a given male in the same way. This would add noise to the data, rendering our results more

Table 1. Associations between haplotype-specific fertilization success and MHC divergence of egg and sperm haplotypes.

	Initial data			Combined data		
Haplotype-specific fertilization success	*N*	Chi-square	*P*-value	*N*	Chi-square	*P*-value
A) Among all four competing sperm haplotypes	73	12.6 (1,67)	0.0004	141	21.8 (1,134)	3.1×10^{-6}
B) Between a male's two sperm haplotypes (focal vs. nonfocal haplotype)	32	8.0 (1,27)	0.0046	44	9.0 (1,38)	0.0027

Chi-square values (with term-specific degrees of freedom and residual degrees of freedom of the model) and *P*-values are shown for the model term describing a quadratic association between relative fertilization success of a given combination of egg and sperm MHC haplotypes and their sequence divergence. Using generalized linear mixed-effects models, fertilization success was compared A) across all four competing sperm haplotypes within a female egg haplotype, and B) between the two sperm haplotypes of a male. Results are given for the initial data alone as well as for the combined data from initial and replication experiment.

conservative, but would not bias our fertilization results toward any given MHC haplotype. In addition, each sperm haplotype represents two distinct values of egg-to-sperm MHC divergence, one for each of the two egg MHC haplotypes available. Each sperm will therefore have a different fertilization success with regard to the two egg MHC haplotypes under a scenario of sperm selection for intermediate egg-to-sperm MHC divergence. Nevertheless, to try to increase our confidence in the observed nonrandom fertilization, we performed another test that compared only the two sperm haplotypes within each male, ruling out any effects of potential MHC-independent intermale differences. We note here that similarity of egg-to-sperm-MHC divergence between two sperm haplotypes of different males might generate competition that affects the ratio of potential success of the two sperm haplotypes within each male. However, the influence of this kind of competition would randomly affect in one case the haplotype that would be preferred by an egg, but with the same probability the unpreferred sperm haplotype. This interaction is thus conservative with respect to the predicted result, as it only increases unspecific variation.

When analyzing fertilization success between a male's two sperm haplotypes, randomly defining one of them as the focal haplotype, we again found the quadratic association between egg-to-sperm MHC divergence conferred by the focal sperm's haplotype and its fertilization success (GLMM, $X^2_{1,27} = 8.0$, $P = 0.005$; Table 1, Fig. 3A). This result confirms the pattern found among all four sperm haplotypes, but here our finding of haplotype-specific MHC-dependent sperm selection by eggs is not confounded by potential differences between males.

However, given the novelty of our findings of haplotype-specific gamete selection, we then decided to replicate the experiment to increase the sample size. The fish handling and in vitro fertilizations of the replication experiment were performed by a different experimenter than in the initial experiment, but otherwise used the same experimental protocol and analysis pipeline. Reassuringly, the new data showed the same quadratic association between the egg-to-sperm MHC divergence conferred by a sperm's haplotype and its fertilization success, when tested across all four sperm haplotypes available to a female's egg ($N = 68$; GLMM, $X^2_{1,62} = 10.8$, $P = 0.001$). Testing the same relation between a male's two sperm haplotypes in the replication data alone was not significant ($N = 12$; GLMM, $X^2_{1,7} = 0.7$, $P = 0.41$), likely owing to the small sample size. However, when we combined the data of the initial and the replication experiment, the significance of our previous results increased, both when tested among all four available sperm haplotypes as well as when comparing focal and nonfocal sperm haplotypes within a male (Table 1, Fig. 3A). Removal of a sperm pair whose focal haplotype exhibited extremely low fertilization success (data point in bottom left corner of Fig. 3A) did not eliminate statistical significance (GLMM, $X^2_{1,37} = 4.6$, $P = 0.032$).

It has been argued that quadratic model terms may per se explain more variation than linear terms and thus require additional support to prove an optimal association. To alleviate such concerns, we therefore here present an analysis that is based on the same logic as has been used for demonstrating the optimal number of MHC alleles at the individual level (Aeschlimann et al. 2003; Milinski 2003; Wegner et al. 2003; Kalbe et al. 2009). This analysis is based on an index that we calculate for each combination of an egg haplotype with a male's two sperm haplotypes by subtracting the number of eggs (with the given haplotype) fertilized by the less divergent haplotype from the number of eggs fertilized by the more divergent haplotype. Under a scenario with gamete selection for intermediate egg-to-sperm MHC divergence, this index is therefore predicted to be positive if the mean egg-to-sperm MHC divergence of a male's two sperm haplotypes is below, and negative, if it is above the optimal divergence (for a schematic see inset of Fig. 3B). If a regression line through all these pair-wise differences in egg number has a significant negative slope, there must be an optimum depicted by the zero intercept of the regression line (see (Milinski 2003) for a detailed description of this approach).

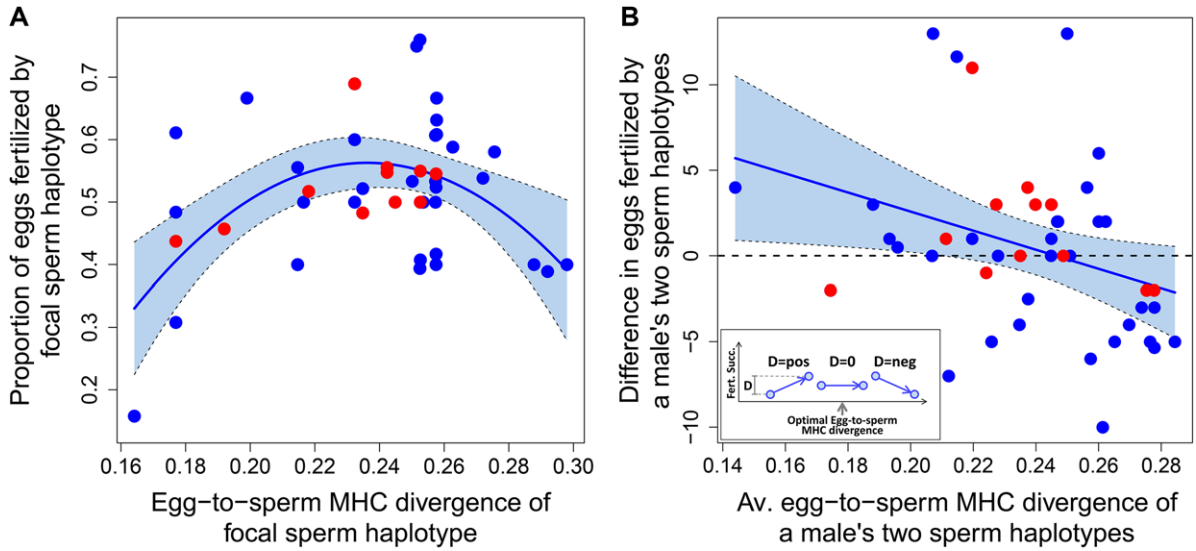

Figure 3. Differential fertilization success of a male's two sperm haplotypes indicates preference for intermediate MHC divergence between egg and sperm.

(A) For each triad combination of female egg MHC haplotype and a corresponding male's two sperm MHC haplotypes ($N = 44$, black arrows in Fig. 1), the proportion of these eggs fertilized by the male's randomly selected focal haplotype over the total number of these eggs fertilized by that male is shown. This proportion is plotted against the MHC IIB amino acid divergence between the egg's MHC haplotype and the focal sperm MHC haplotype (egg-to-sperm MHC divergence). The line and shaded area correspond to a fitted binomial GLMM with a significant quadratic term for MHC haplotype divergence (GLMM, $X^2_{1,38} = 9.0$, $P = 0.003$) and its 95% confidence interval. Blue and red dots mark data from the original ($N = 32$) and the replication experiment ($N = 12$), respectively. (B) For the same triad combinations of female egg MHC haplotype and the corresponding male's two sperm MHC haplotypes ($N = 44$), the mean egg-to-sperm MHC divergence of the two sperm haplotypes is plotted against the difference D in number of eggs fertilized by the more divergent sperm haplotype and the less divergent sperm haplotype. The inset shows the calculation of D and its expected sign if sperm with an intermediate egg-to-sperm MHC divergence were favored. The observed negative correlation (Kendall's tau = 0.27, $P = 0.013$) confirms that expectation and the zero intercept (dotted line) approximates the optimal MHC divergence between egg and sperm haplotypes. Coloring as in panel A.

In agreement with this prediction, we found a significant negative correlation between the mean egg-to-sperm MHC divergence of the two sperm haplotypes within a male and their difference in number of fertilized eggs, both in the initial data ($N = 33$, Kendall's tau = –0.25, $P = 0.045$) as well as in the combined data ($N = 45$, Kendall's tau = –0.25, $P = 0.018$; Fig. 3B), thus confirming the intermediate optimum. This dual-evidence approach depicted in Figs. 3A, B, here performed at the haplotype level, conceptually follows the analysis for whole genotypes by Wegner et al. (2003). It reveals that eggs were indeed preferentially fertilized by the male's sperm haplotype that provided the more intermediate (optimal) MHC divergence.

As the main goal of this study was an investigation of MHC-specific effects at the haplotype level, we had excluded egg-sperm combinations with identical MHC haplotypes from our analyses, as described in the introduction. The concern is that in those cases the two gametes are likely to also be identical at other genes in the chromosomal vicinity of the MHC genes so that any differential fertilization could not be exclusively attributed to the MHC genes. However, as several previous studies have reported postcopulatory fertilization advantage for MHC-similar mates at the individual level, we took advantage of our dataset and also tested for this effect. Interestingly, when comparing fertilization success of males that shared an MHC haplotype with the female to males sharing no haplotype with the female, our data showed a similar fertilization advantage for MHC-similar mates (GLMM, $X^2_{1,43} = 5.4$, $P = 0.02$). This effect was masked in our main analyses because of the exclusion of homozygous egg-sperm combinations. When including these combinations in our analyses, sperm with identical MHC haplotype to an egg indeed appear to be favored to some extent (GLMM, $X^2_{1,148} = 5.7$, $P = 0.017$), independent of the above reported preference for intermediate MHC divergence in nonidentical gamete combinations (quadratic term remains significant: GLMM, $X^2_{1,148} = 15.8$, $P = 7.2 \times 10^{-5}$). However, as this homozygous effect cannot be confidently attributed to the MHC genes alone and furthermore contrasts with the observed diversity at the MHC genes in natural populations, this phenomenon requires additional research to resolve the involvement of MHC

and/or other genes in the MHC region (e.g., olfactory receptor genes; Ziegler et al. 2010).

Discussion

Fertilization success during postcopulatory sexual selection can contribute substantially to a male's overall reproductive success (Pischedda and Rice 2012). Consequently, research on postcopulatory fertilization success has spurred the discovery of intriguing mechanisms of intermale sperm competition (Wedell et al. 2002; Fisher and Hoekstra 2010; Immler et al. 2011; Yeh et al. 2012; Crean et al. 2016; Kekäläinen and Evans 2017). Likewise, cryptic female choice for sperm of compatible mates in an intermale competition scenario has been demonstrated, however, only at the individual level (Olsson et al. 1996; Clark et al. 1999; Yeates et al. 2009; Løvlie et al. 2013; Firman and Simmons 2015; Gasparini et al. 2015), that is the sperm of more compatible males is preferred to sperm of less compatible males. Our results extend these previous findings to a new and more sophisticated level by providing empirical evidence for the existence of a mechanism of haplotype-specific cryptic female choice for compatible sperm not only among sperm from different males but also between sperm from a single male. During in vitro fertilization, eggs of three-spined sticklebacks were preferentially fertilized by those sperm of a male that carry the MHC haplotype with which the genotype of the resulting zygote is closer to an intermediate individual MHC diversity.

This empirical confirmation of the hypothesized mechanism of haplotype-specific preference for intermediate MHC divergence adds a new dimension to earlier findings of mate selection for intermediate (i.e., optimal) MHC diversity at the individual level (Reusch et al. 2001; Aeschlimann et al. 2003; Milinski et al. 2005; Forsberg et al. 2007). An intermediate MHC diversity is known to provide optimal immunocompetence in resistance to pathogens due to an optimized trade-off between broad antigen presentation by MHC molecules and a large repertoire of reactive T cells (which is negatively affected by increasing MHC diversity; Nowak et al. 1992; Woelfing et al. 2009; Chappell et al. 2015). The fitness advantage of such optimal MHC diversity has been confirmed empirically in three-spined sticklebacks (Wegner et al. 2003; Wegner et al. 2008; Kalbe et al. 2009) and other species (Bonneaud et al. 2004; Madsen and Ujvari 2006; Kloch et al. 2010; Hawley and Fleischer 2012), rendering sexual selection for optimal offspring MHC diversity highly advantageous (Milinski 2006; Woelfing et al. 2009). This previously reported evidence, however, so far demonstrated only precopulatory female mate choice for specific males, which still suffered from the phenotypically unpredictable lottery effect of random Mendelian segregation of MHC haplotypes between gametes. In contrast, our present results suggest such selection mechanisms at the haploid level and provide a unique mechanism to counteract this lottery effect, favoring offspring genotypes with the most optimized immunocompetence. Eventually, this mechanism of sexual selection, in combination with additional processes such as pathogen-mediated negative frequency-dependent selection, contributes to the maintenance of the frequently observed high allelic diversity at the MHC in natural populations (Milinski 2006; Eizaguirre et al. 2012).

The detection of such potentially subtle effects as sperm selection by eggs studied here requires both a thorough experimental approach and fine-scaled parameter estimates. Due to limitations in technological development, earlier studies on the evolutionary significance of MHC genetics often had to rely on counting the number of distinct alleles to characterize an individual's MHC diversity. However, distinct MHC alleles can differ by as little as one amino acid to as much as 27 amino acids or about 40% of the so-called antigen-binding groove in which pathogen-derived antigens are presented to the immune effector cells (Lenz et al. 2009a). Recent computational analyses showed that the extent to which two MHC alleles differ in their coding sequence is directly correlated with the extent to which the corresponding MHC molecules differ in their functional antigen-binding capacity (Lenz 2011; Pierini and Lenz 2018). At the same time, new advances in genotyping and sequencing technology provide unprecedented genotype information at the sequence level and thus allow for more fine-scaled analyses than merely distinguishing between distinct alleles (Babik et al. 2009; Lenz et al. 2009a). Consequently, individual MHC diversity is increasingly often being estimated by quantifying the sequence divergence among an individual's MHC alleles. This new measure of individual MHC diversity, called MHC divergence, is expected to more directly capture an individual's MHC-dependent immunocompetence and has proven successful in recent studies, including selection for and advantage of intermediate individual MHC divergence in different species (e.g., Forsberg et al. 2007; Lenz et al. 2009b; Evans et al. 2012; Lenz et al. 2013). Here, we followed this development, using the average sequence divergence between parental MHC IIB haplotypes as an estimator for individual MHC diversity of the resulting offspring, and thus were able to detect patterns of selection that may have remained elusive without these technological advancements. We can here only speculate as to why three-spined sticklebacks exhibit MHC-dependent gamete selection while this could not be shown in a recent experiment on Atlantic salmons (Promerova et al. 2017). One possible explanation is that here we allowed for a larger diversity of sperm haplotypes by using sperm from two different males, while eggs in Promerova et al. (2017) were only provided with sperm from one male. Haplotype-specific gamete selection may be more pronounced when a wider range of different haplotypes is available. In line with this explanation, a previous study found MHC-dependent fertilization success in

Atlantic salmon when eggs were exposed to sperm from two different males, although it is unclear whether that result was driven by cryptic choice at the gamete level or at the individual level (Yeates et al. 2009).

While sperm selection for MHC complementarity has been hypothesized repeatedly (Wedekind 1994; Tregenza and Wedell 2000; Birkhead and Pizzari 2002; Ziegler et al. 2005; Milinski 2006), the exact molecular mechanism for potential MHC-based gamete interactions is still debated. To allow for sperm selection with respect to MHC II, the first prerequisite is expression of these molecules on the sperm surface. While some studies have found no MHC expression, others reported MHC I and/or MHC II molecules on mature sperm cells (reviewed in Hutter and Dohr 1998; Fernandez et al. 1999; Dadoune et al. 2004). It therefore remains to be investigated whether the described effect is directly mediated by MHC II molecules or by other loci of the extended MHC region as suggested by Ziegler et al. (2005, 2010). Either way, a recent study showed that the micropyle of fish eggs, an opening in the egg coat through which sperm enter the egg for fertilization, appears to carry specific molecules that are involved in attracting sperm toward the micropyle opening and whose removal significantly reduces fertilization success (Yanagimachi et al. 2013). Tests showed that this mechanism was species-specific, suggesting a certain specificity of the molecular mechanism involved and highlighting a potential way to preferentially guide MHC-complementary sperm to the micropyle opening. The second prerequisite for haplotype-specific sperm selection is the haploid expression of MHC genes during sperm development. While most transcripts are shared among spermatids via cytoplasmic bridges, there is now increasing evidence that a significant number of genes are not shared but instead expressed from the haploid genome of mature sperm (Joseph and Kirkpatrick 2004; Immler 2008). Indeed, a recent study found evidence for haploid selection in zebrafish, presumably based on genetic differences among haploid gametes (Alavioon et al. 2017). The self-incompatibility mechanisms in some plants and ascidians, which prevent self-fertilization, also require expression of the haploid gamete allele (Takayama and Isogai 2005; Harada and Sawada 2008), indicating that such fine-tuned mechanisms have evolved multiple times.

While here, the exact mechanism is still under investigation, the selective advantage is intriguing: Eggs selecting compatible sperm, for which we show evidence here, allows for correcting both precopulatory "mistakes" arising from phenotypically unpredictable allele segregation among gametes during meiosis and noncomplementary sperm from nonchosen sneaker males that fertilize clutches almost simultaneously with the chosen male. Oocytes preferring sperm with complementary immunogenes therefore gain an evolutionary advantage in the highly dynamic arms race between host and parasites.

Material and Methods

IN VITRO FERTILIZATION EXPERIMENT

For the initial experiment, parental sticklebacks were caught from the lake Großer Plöner See (Northern Germany) in May 2006. In the lab, they were gradually brought to summer conditions with food provision ad libitum. Males were kept individually in 10 l tanks and provided with artificial nesting material, whereas females were kept in small groups of 4–5 individuals. Only gravid females ($N = 11$) and reproductively active males ($N = 22$) maintaining a nest were used and randomly selected for the experiment. With each female and male carrying two MHC IIB haplotypes, respectively, this led to 88 combinations of egg and sperm haplotypes, and each female egg being confronted with four different sperm MHC IIB haplotypes (Fig. 1). We did not control for relatedness among paired individuals. However, given that these were wild-caught fish from a very large lake population, it is highly unlikely that the selected individuals were related. Furthermore, genome-wide relatedness is not expected to correlate with MHC sequence divergence and relatedness does not appear to have a significant influence on fertilization success in sticklebacks (Mehlis et al. 2015). Eggs were carefully stripped from females by applying gentle abdominal pressure, while sperm was extracted from dissected testes of males and collected in 300 μl Hank's balanced salt solution (HBSS). Eggs were fertilized in vitro under competition with a sperm mix obtained in equal volumes (30 μl sperm/HBSS) from two different males. The milt/HBSS volume was selected as males are assumed to release about 5% of their sperm store in a single mating (Zbinden et al. 2001). Since testicular sperm were used here, the sample potentially also contained immature, nonfertilizing sperm so 10% of the total sample volume was used to approximate the absolute number of mature sperm from a natural ejaculate. This amounted to an average of approximately 2,583,135 mature sperm cells from each male. Precautions were taken to avoid contamination from water or feces in order to prevent premature activation of the gametes. Tank water was added to the eggs after provision of sperm and eggs were left for 15 minutes by which time fertilization was complete. During this time, the total number of sperm cells of both males was determined from sperm/HBSS subsamples using a modified form of the standard cell dilution assay (SCDA; Pechhold et al. 1994). This technique allows for distinguishing cellular debris and dead cells from viable cells (Scharsack et al. 2004).

After fertilization, eggs were kept in aerated 1 l glass jars for rearing. Just before hatching (~6 days), the eggs were frozen individually at –20°C for genetic analyses. All eggs were fertilized and mortality during egg rearing was negligible (<2% for all clutches).

For the replication experiments, fish were caught in early 2015 from the same lake population and handled/processed in

the same way as described above. The same criteria for reproductive maturation as above were employed to produce a total of 12 new clutches, using 12 females and 22 males (sperm of one male was used thrice). The fraction of undeveloped eggs was again low with a median of 2 (±5 SD) percent, comparable to the initial experiment and as expected from in vitro fertilization.

All animal experiments described were approved by the Ministry of Agriculture, the Environment and Rural Areas, Schleswig-Holstein, Germany.

MHC IIB GENOTYPING AND HAPLOTYPE SEGREGATION

For the initial dataset, genomic DNA was extracted using the DNATissueHTS kit (Invitek, Germany). MHC IIB haplotypes in the three-spined stickleback carry a variable number of tightly linked loci (carrying 1–3 different alleles; Lenz et al. 2009a). Here, reference strand-mediated conformation analysis (RSCA) was employed for genotyping the exon 2 of all MHC IIB loci per haplotype (Lenz et al. 2009a). Out of a median total clutch size of 119 ± 21 SD in our experiment, we attempted to genotype 96 randomly picked eggs. A total of 890 embryos (median: 83 ± 11 SD, leading to an average genotyping rate of 70% per clutch) were successfully typed for their MHC IIB genotype, providing a robust representation of the genotype distribution in the clutches. The parents were genotyped in the same way to identify the parental origin of each MHC haplotype. No recombination event was observed in the offspring genotypes. In case of haplotype identity within or between males, equal fertilization success was assigned to each haplotype. Random segregation of parental MHC IIB haplotypes during meiosis was verified by running exact binomial tests on the frequency of each parent's two haplotypes in their offspring (all FDR-corrected *P*-values > 0.05), thus rejecting the possibility of MHC-dependent skews during gamete production (e.g., through meiotic drive mechanisms). Importantly, this result does not preclude MHC-dependent gamete selection. A male's two sperm haplotypes each could still preferentially fertilize the more complementary of a female's two egg haplotypes, even though they overall fertilize the same number of eggs. For females, this test was directly informative about the segregation of MHC haplotypes during meiosis.

In combination with a library of known alleles, RSCA genotyping allows identification of MHC IIB alleles to the sequence level (Lenz et al. 2009a). The obtained sequence information was used to calculate the haplotype divergence Div_{MHC} of male to female MHC IIB haplotypes as the average amino acid *P*-distance (proportion of divergent sites over all sites) between all possible allele pairs of the two parental haplotypes (Forsberg et al. 2007; Evans et al. 2012) according to the following equation:

$$Div_{MHC} = \frac{2^* \sum_{i=1}^{a_p-1} \sum_{j=i+1}^{a_p} d_{A_i A_j}}{a_p{}^* \left(a_p - 1\right)}$$

where a_p is the number of parental alleles and *dAiAj* is the amino acid *P*-distance between the ith and the jth parental allele. This equation accounts for a variable number of alleles among the parental haplotypes by averaging the pairwise allele divergence over the number of pairwise allele comparisons. Div_{MHC} is called "*egg-to-sperm MHC divergence*" throughout the manuscript.

In the initial experiment, one of the 11 females turned out to be homozygous, yielding only one type of egg MHC haplotype for analysis of fertilization success. Also, three of the 22 males were homozygous, so that their relative fertilization success was determined by only one sperm haplotype. The rational for this is that counting the two identical haplotypes as two separate observations/data points would represent pseudoreplication. Technically, this step does not actually remove sample information, but merges them. However, this does lead to a reduction in data points. As explained in the main text, we required for our analyses that egg and sperm haplotypes differ in their MHC, in order to guarantee that effects could be linked directly to the MHC genes. This resulted in a total number of 73 egg-sperm combinations from the initial experiment. For the focal/nonfocal haplotype analysis within males, we also had to remove all homozygous males (only one MHC haplotype) and exclude data from males that shared one or both MHC haplotypes with the other competing male, as this led to the same embryo genotypes and male-specific fertilization success could thus not be unequivocally determined. This would have impeded the main goal of this "within-male" analysis by introducing various male-specific and potentially confounding factors. This resulted in a total number of 32 focal/nonfocal sperm pairs from the initial experiment. See Fig. S2 for a detailed listing of excluded samples and rationale.

Samples from the replication experiments were processed in the same way as above, except that genomic DNA was extracted using the Qiagen DNAeasy Kit (Hilden, Germany). This time we aimed to genotype all eggs of a given clutch. The 12 clutches yielded a total of 1397 eggs, with a median clutch size of 122.5 (±23 SD) eggs. A total of 1267 (90%) developing embryos could be genotyped successfully, yielding a median of 104 (± 19 SD) genotyped eggs per clutch. The randomly selected fish of this replication cohort carried a slightly less diverse set of MHC genotypes, resulting in a significant reduction of egg-sperm combinations that could be used for analyses: Three of the 12 females turned out to be homozygous for their MHC

IIB genotype, and four of the 23 males were also homozygous. This resulted in a total number of 68 egg-sperm combinations from the replication experiment. For the focal/nonfocal haplotype analysis, further removal of males with shared MHC haplotypes (see above) resulted in a total number of only 12 focal/nonfocal sperm pairs from the replication experiment. See Fig. S2 for a detailed listing of excluded samples and rationale.

STATISTICAL ANALYSES AMONG ALL FOUR AVAILABLE SPERM HAPLOTYPES

We initially employed a nonparametric approach based on Monte Carlo sampling (Metropolis and Ulam 1949) to test our observed data for deviations from random fertilization success. This Monte Carlo simulation approach allowed us to combine the observed fertilization patterns across clutches without the pitfalls of multiple testing. First, we focused on the question whether the four sperm haplotypes available to each given egg haplotype obtained fertilization success compatible with random fertilization. For this we simulated the entire set of experimental fertilization events (same number of clutches, same females, males, and same number of eggs per female MHC haplotype), with the key exception that in the simulations the sperm that fertilizes each egg is picked randomly (with a probability proportional to the relative sperm number of its male) from the four sperm MHC haplotypes (two from each of the two males) that are actually available to the given egg from the two males of the given one female–two males combination. This creates a simulated dataset of random fertilization success after accounting for differences in sperm number between the two males, but all else being equal to the observed data. We then determined for each sperm haplotype in the simulated data how many eggs of a given female haplotype it "fertilized" and calculated the deviation from the fertilization success expected purely based on differences in sperm number. Eventually we calculated the mean of this deviation across all sperm haplotypes of the entire simulated dataset. We then repeated this simulation 1000 times to obtain a distribution of average deviation from fertilization success that could be expected under random fertilization, accounting for possible stochasticity given the observed number of fertilization events. Comparing the same average deviation from the observed dataset to this distribution of simulated values allowed us to estimate the significance of the observed nonrandom fertilization.

We then aimed to identify directionality in the nonrandom fertilization by estimating the likelihood of obtaining the observed egg-to-sperm MHC divergence distribution (mean and variance) of the most successfully fertilizing ("preferred") sperm haplotype for each given egg haplotype under a random fertilization scenario. The likelihood was obtained using a Monte Carlo sampling procedure (Metropolis and Ulam 1949) with 1000 simulation runs, randomly sampling one of the four available sperm MHC haplotypes for each of the female egg haplotypes and thus mimicking random fertilization.

To test whether the more successful egg-sperm combinations exhibited an intermediate egg-to-sperm MHC divergence, we ranked the four sperm haplotypes available to a given egg according to their fertilization success with this egg haplotype. In case of equal fertilization success, we picked one randomly. We then calculated the deviation (absolute difference) of their egg-to-sperm MHC divergence to a hypothetical optimal level of individual MHC divergence. This hypothetical optimal MHC divergence was defined as the mean individual MHC IIB divergence of the parental population (0.2356 ± 0.0490 SD; calculated from the combination of the two MHC haplotypes in an individual, making it exactly equivalent to the egg-to-sperm MHC divergence). This value was obtained from an independent dataset of randomly wild-caught individuals ($N = 90$) from the same cohort (Eizaguirre et al. 2009) and was virtually identical to the mean individual MHC IIB divergence of the wild-caught parental fish of the present dataset (0.2353 ± 0.0395 SD; $N = 33$).

We then employed a model-based approach to explore the association between egg-to-sperm MHC divergence and relative fertilization success in more detail and without making a-priori assumptions about the exact location of the optimal MHC divergence. Specifically, we used a generalized linear-mixed effects model (GLMM, lme4 package in R) with binomial error distribution and logit link to test for a quadratic association between relative fertilization success of a given egg-sperm combination and its egg-to-sperm MHC divergence. The dependent variable was the number of eggs of that egg-sperm haplotype combination over the total number of eggs with that egg haplotype (i.e., fertilized by the other three available sperm haplotypes). Linear and quadratic terms for the egg-to-sperm MHC divergence were included as fixed effects, and female haplotype, nested within clutch identity, was used as a random effect to account for pseudoreplication of female MHC haplotypes. In this analysis of fertilization across the four sperm haplotypes from the two males, we also included males' sperm number as a fixed effect, as sperm number has the potential to affect fertilization success in competition scenarios. Expectedly, the number of sperm cells provided by a given male did not correlate significantly with egg-to-sperm MHC divergence of the observed zygotes (Spearman's rho $= 0.12$, $P = 0.28$). When analyzing the combined data from both experiments, we also included the year of the experiment as a random effect in the model to account for variation between the two experiments. To account for possible differences between males, we then continued with the analyses by comparing fertilization success only between the two haplotypes of each male separately, as described in the next section.

STATISTICAL ANALYSES FOCUSING ON THE TWO SPERM HAPLOTYPES WITHIN A MALE

For a direct analysis of the fertilization success of the two sperm haplotypes within males, we randomly chose one of them as the focal haplotype (see Gage et al. 2004). Eggs fertilized by the focal haplotype over eggs fertilized by focal and nonfocal haplotypes were used as response variable in a GLMM with binomial error and logit link. Linear and quadratic terms for the MHC divergence of focal haplotype to the respective egg haplotype (egg-to-sperm MHC divergence) were included as fixed effects and female haplotype nested within clutch identity was used as a random effect to account for pseudoreplication. When analyzing the combined data from both experiments, we also included the year of the experiment as a random effect in the model to account for variation between the two experiments. Terms were individually removed from the model to estimate their explanatory significance. To test for an association between mean egg-to-sperm MHC divergence and the difference in fertilization success of a male's two sperm haplotypes, we used a nonparametric Kendall correlation that is most suited to account for ties in the data. We subtracted the number of eggs with a given female MHC haplotype fertilized by the sperm providing lower egg-to-sperm MHC divergence from the number of eggs fertilized by the sperm providing higher MHC divergence. Analyses were performed in R 2.14.2 (R Development Core Team 2012) and required packages (car (Fox and Weisberg 2010), lme4 (Bates and Maechler 2010), languageR (Baayen 2010)). All *P*-values are two-tailed.

AUTHOR CONTRIBUTIONS

M.M., S.E.Y., and T.L.L. conceived the study. S.E.Y., N.H., and I.E.S. performed in vitro experiments. T.L.L. performed genotyping and analyzed data. T.L.L., M.M., and N.H. interpreted the results. T.L.L. and M.M. wrote the manuscript with input from all authors.

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DATA ARCHIVING

The analyzed dataset, including all data sheets needed to replicate statistical tests and figures, is available from the Dryad repository. The doi for our data is https://doi.org/10.5061/dryad.bp11pv2.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Deviation from random fertilization success.
Figure S2. Detailed explanation of data filtering for main analyses.