Ecology can play a major role in species diversification. As individuals are adapting to contrasting habitats, reproductive barriers may evolve at multiple levels. While pre-mating barriers have been extensively studied, the evolution of post-mating reproductive isolation during early stages of ecological speciation remains poorly understood. In diverging three-spined stickleback ecotypes from two lakes and two rivers, we observed differences in sperm traits between lake and river males. Interestingly, these differences did not translate into ecotype-specific gamete precedence for sympatric males in competitive in vitro fertilization experiments, potentially owing to antagonistic compensatory effects. However, we observed indirect evidence for impeded development of inter-ecotype zygotes, possibly suggesting an early stage of genetic incompatibility between ecotypes. Our results show that pre-zygotic post-copulatory mechanisms play a minor role during this first stage of ecotype divergence, but suggest that genetic incompatibilities may arise at early stages of ecological speciation.

1. Introduction

Studying recently diverged species has provided us important insights into the processes and mechanisms of speciation [1]. Ecological speciation defines the evolution of reproductive barriers caused by divergent natural selection in different ecological niches or habitat types [2], leading to the formation of ecological demes (ecotypes). Distinct mechanisms can generate and maintain reproductive isolation between ecotypes such as local adaptation, selection against hybrids or preference for conspecific mates [2–4]. Surprisingly, to date, only a limited number of studies have examined the importance of post-copulatory processes in the context of incipient ecological speciation even though post-copulatory traits can initiate, reinforce and maintain reproductive isolation [5,6]. Under divergent ecological selection regimes, males may, for instance, evolve different sperm characteristics (i.e. morphology and velocity) which may facilitate conspecific female fertilization or increase sperm competitiveness in the local environment [7]. Furthermore, gametic incompatibilities could occur, based on allelic or genotypic differences, between partners from different populations [5].

Three-spined sticklebacks (Gasterosteus aculeatus spp., L.) have become a model organism in ecological speciation [8]. Ecological contrasts between habitats have led to divergent selection on various traits such as morphology, feeding behaviour and mating systems [3,9,10]. Sticklebacks are external fertilizers that experience sperm competition in the context of alternative male mating strategies, such as sneaking, and thus facilitate the investigation of post-copulatory reproductive barriers [11]. Particularly, externally ejaculated
spermatozoa are directly confronted with habitat-specific ecological conditions during spawning which provides the potential to evolve different sperm traits in contrasting habitats [7].

The system of lakes and rivers in northern Germany is a young post-glacial system where different stickleback ecotypes can be found [12]. In this system, local adaptation [4] and female preference for sympatric males [13] contribute to pre-copulatory reproductive barriers. Here, we (i) evaluate sperm traits in replicated populations and (ii) experimentally test for ecotype-specific gamete precedence between lake and river stickleback ecotypes.

2. Material and methods
(a) Study system
Three-spined sticklebacks were caught from two independent pairs of geographically connected lake and river populations, representing two drainage systems in northern Germany (electronic supplementary material, S1). After 20 weeks under standardized winter-like conditions (8 h day : 16 h night; 6 °C) and six weeks in spring-like conditions (12 h : 12 h; 12 °C), fish were isolated singly into 16 l tanks under summer conditions (16 h : 8 h; 18 °C). There, to build a nest, males were provided with artificial nesting material [14].

(b) Experimental design
We performed in vitro sperm competition trials following a full-factorial design involving a female, a sympatric male and a ‘competing’ male. This competing male was either from the same ecotype as the female or from a different ecotype. Three to six such triads were conducted for each of the 16 possible population combinations (electronic supplementary material, S2). This resulted in 64 independent sperm competition trials (33 within-ecotype and 31 between-ecotype). In trials with two sympatric males, we randomly declared one male as the focal male. Individuals were used once only.

(c) Sperm measurements and competition trials
All males were presented with a ripe female within 24 h prior to dissection and showed active reproductive behaviour, for example, nest gluing [14]. Fish were sacrificed by an incision in the brainstem prior to dissection. After dissection, sperm was isolated by mashing the entire testes in 900 μl of Hank’s balanced salt solution. We measured sperm concentration as well as curvilinear, straight-line and average-path velocities using computer-assisted sperm analysis (electronic supplementary material, section Methods). Each egg clutch was carefully stripped into a dry Petri dish and fertilized by a mixture containing 20 μl of sperm solution from each male in 5 ml of fresh water. Differences in ejaculate traits thus reflect natural conditions. Fertilized eggs were reared under controlled conditions with oxygenated water. Five days after fertilization, eggs were counted and categorized into unfertilized (no visible yolk, undeveloped (dead yolk) and developed eggs [15]. An insufficient number of fertilized eggs in seven clutches (four within-ecotype, three between-ecotype) led to a total of 57 independent trials. We genotyped all fertilized eggs (Ntotal_egg = 2508) and parents (Nadult = 171) at five microsatellite loci and identified the most likely sire using CERVUS v. 3.0.3 (Field Genetics Ltd [16]; electronic supplementary material, section Methods).

(d) Statistical analyses
Relative paternity and the proportion of undeveloped eggs were normalized using arc-sinus and log transformation, respectively.

First, we analysed differences in sperm traits (concentration and sperm velocity) between ecotypes using ANCOVAs with ecotype, drainage system and their interaction as cofactors and different paternity as covariate (lm function in R). Second, we performed an analysis of covariance (lm function in R) on the proportion of eggs sired by the sympatric male (relative paternity). The full model included female ecotype and the type of competition (within-ecotype versus between-ecotype) as factors, and relative spermatozoa concentration, relative velocity (sympatric/competitive) and their two-way interaction as covariables. Owing to multicollinearity, we corrected the explanatory covariables using the residuals of sperm concentration on tests mass and ecotype identity and the residuals of sperm velocity (PC1 values; electronic supplementary material, section Methods) on testes mass, total sperm concentration and ecotype identity. The best-fitting model was selected using an AIC-based backward selection procedure (stepAIC function in R). We similarly tested for variation in the proportion of undeveloped eggs in relation to paternity and the type of competition. All statistical tests were conducted in R v. 3.0.3 [17].

3. Results
We found that sperm velocity was significantly higher for lake males than river males (F1,141 = 9.07, p = 0.003; electronic supplementary material S3 and figure 1a), whereas river fish showed higher sperm concentration (F1,141 = 9.52, p = 0.002; electronic supplementary material, S3 and figure 1b). These differences suggest ecotype-specific sperm characteristics even though their extent varied between the population pairs as indicated by significant interactions between ecotype and drainage system (electronic supplementary material, S3).

These sperm characteristics did not translate into significant sperm precedence for sympatric males (F1,57 = 0.11, p = 0.744, table 1). Our sample size provided sufficient statistical power to detect sperm precedence of magnitudes similar to those reported in other species pairs (h = 0.835, power > 0.99; electronic supplementary material, S4). Both relative velocity and relative spermatozoa concentration between competing males were strong predictors of paternity (velocity: F1,57 = 10.91, p = 0.002; concentration: F1,57 = 10.12, p = 0.003).

Interestingly, we found that the origin of the competing male significantly affected the proportion of undeveloped eggs in interaction with the relative paternity of the sympatric male (F2,57 = 4.160, p = 0.021): the relationship between paternity and the proportion of undeveloped eggs was significant only when sperm competition involved males from different ecotypes (between-ecotype: r = 0.442, 95% CI = 0.09–0.69,
Table 1. Effects of sperm characteristics and ecotype origin on (a) paternity of sympatric male and (b) proportion of undeveloped eggs. Statistical table showing results of linear models. Only variables included in the best fitted model are shown. (d.f., degrees of freedom; S.S. sum of squares). Significant effects are printed in italics.

<table>
<thead>
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<th></th>
<th>d.f.</th>
<th>S.S.</th>
<th>F-value</th>
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<td>(a) Paternity of sympatric male</td>
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<td>(b) Proportion of undeveloped eggs</td>
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Figure 2. Relationship between paternity of the sympatric male and the proportion of undeveloped eggs. Filled circles and solid regression line (significant) correspond to between-ecotype trials, and open circles and dashed line correspond (non-significant) to within-ecotype trials.

$p = 0.016$; within-ecotype: $r = 0.183$, 95% CI = $-0.18–0.51$, $p = 0.32$ and figure 2).

4. Discussion

Investigating post-copulatory reproductive isolation between three-spined stickleback ecotypes, we found differences in sperm traits between males from lake and river. Particularly, river males showed higher sperm concentration and slower spermatozoa speed than lake males. Differences in these sperm traits may stem either from neutral processes or from contrasting abiotic and biotic ecological pressures between habitats known to affect such phenotypes (e.g. temperature [18] and parasites [19]). However, despite variation in the extent of sperm trait differentiation between two independent drainage systems, the fact that we found a parallel pattern suggests an ecological origin of these differences.

Our experiment confirmed that sperm number and sperm velocity are major predictors of paternity in fishes, particularly during sperm competition [20]. Although sperm traits diverged between ecotypes, these differences did not lead to biased paternity for sympatric males in between-ecotype competition trials and thus did not translate into post-copulatory pre-zygotic reproductive isolation. We can hypothesize that high sperm concentration in lake males and high sperm speed in river males might act in a compensatory manner in competitive situations, leading to balanced paternity. In addition, pre-copulatory reproductive isolation is relatively strong in this system, which may decrease selection for sympatric sperm precedence as a reproductive barrier [5,13].

Interestingly, however, we found a positive correlation between the proportion of undeveloped eggs and paternity of the sympatric male in clutches where males from different ecotypes competed for fertilization. In other words, with more eggs failing to develop, the remaining developing eggs were more likely to have been sired by the sympatric male. The fact that this correlation could not be observed in competition trials between ecologically equivalent (same ecotype) males suggests that the developmental failure could be due to emerging Dobzhansky–Muller-type genetic incompatibilities between the diverging stickleback ecotypes [21,22]. Such reproductive barriers at the zygote stage have been shown in many taxa, but so far mostly between distinct species with no gene flow [23,24]. Unfortunately, genotyping of the undeveloped eggs, which would be necessary to confirm the above hypothesis, was impossible owing to the low DNA concentration and quality. Therefore, further investigation is required to confirm this observation.

Altogether, our results show that reproductive isolation in response to ecological adaptation does not necessarily arise at all levels simultaneously. The suggested genetic incompatibility, in turn, may have arisen as a by-product of reduced gene flow, driven by forces such as mate choice and local adaptation, in an otherwise open system [6]. If confirmed by further experimental effort, this would suggest a role for genetic incompatibilities in reproductive isolation between contrasting ecological habitats already at an early stage of speciation.

Ethics statement. All experiments were approved by the Ministry of Energy, Agriculture, the Environment and Rural Areas, Schleswig-Holstein, Germany.

Data accessibility. Data are available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.68t78.

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