Electronic appendix to:

**Sperm numbers vary between inter- and intra- population matings of the grasshopper *Chorthippus parallelus***

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2. MATERIAL AND METHODS

Rearing

In the hybrid zone in SE France, nymphs were collected from P and E populations sites (Puyvalador and Eyne (PU, EY) (Butlin & Hewitt 1985) and transported to a laboratory in the zone at Saillagouse. They were reared in large (20 x 40 x 50 cm) gauze topped cages. Every morning, newly eclosed adults were collected. Separated by sex, individuals were transferred into smaller cages (15 x 15 x 25 cm). Individuals were moved between cages by applying negative air pressure (for collecting) or positive air pressure (for releasing) through a glass tube attached to an aspirator. This procedure is unlikely to mask the surface cuticular hydrocarbons which are known to differ between populations and with age (Tregenza et al. 2000b).

Freshly cut grass blades of *Dactylis glomerata* were offered at 2-3 day intervals. Of each eclosion cohort group, between ten and twenty individuals were placed in a cage to minimise the effects of competitors on strategic ejaculation (Reinhardt 2001). The shelves with the cages were lighted and heated by a halogen spotlight. The temperature during the mating protocols was between 29 and 30.5°C.

Mating protocol

Individuals were randomly allocated to either sympatric or allopatric matings and to one of four sperm storage periods (0, 5, 7 or 10 days), scattered around the half-life of an ejaculate in the spermatheca of *C. parallelus* females (ca. 8 days: Reinhardt et al. 1999).

Females aged 4-6 days were daily offered a male in the smaller cage type. Copulation durations were determined to the nearest two-minute interval. If matings did not occur within three to four hours individuals were returned to their original cage. Because such refusals
were more common in P than E males (3.0 vs. 1.9 times, p = 0.049) and there was an interaction between male and female population (p = 0.027) (see also Ritchie et al. 1989) individuals at mating can be expected to differ in age. The resulting potential bias of male and female age on ejaculate size was circumvented by using male and female age as covariates in the model.

**Sperm counts**

After mating females were immediately placed individually into foam-topped *Drosophila* culture tubes (2cm diameter, 8cm height). Within 30 minutes the entire spermathecal tract was removed as a whole and placed into an Eppendorf tube containing 1ml of distilled water. The spermathecal tract which contained the spermatophore was ruptured by a pair of scissors and homogenized by drawing the solution 30 times through a Pasteur pipette. One drop of the solution was pipetted onto a haemocytometer and the number of sperm heads in the nine inner squares (1.1 µl) counted (Reinhardt *et al.* 1999; Reinhardt 2001) and multiplied by the dilution.

All matings resulted in successful sperm transfer. Potential female manipulations by moving sperm between spatial compartments or by ejecting the spermatophore (e.g. Reinhardt & Meister 2000) were prevented by counting the sperm of the entire spermathecal tract and within less than 30 minutes after copulation. Therefore, sperm counts at storage level zero represent ejaculate size (Reinhardt 2001).

**Sperm storage**

Females assigned to storage treatment were kept individually in cages of the smaller type with food and sand-filled cups for oviposition provided. In most grasshopper species oviposition cannot be prevented by withholding oviposition substrate. Instead the number of egg clutches laid during the storage period was recorded. Because the number of clutches differed between crosses (sample size): ExE: 1.6 ± 1.0 (13), PxP: 0.3 ± 0.8 (6), ExP: 0.7 ± 0.9 (4), and PxE: 0.4 ± 0.8 (12) (F_{3,31} = 4.54, P = 0.009) the number of egg pods was included as a covariable in the model. On the assigned day, the number of sperm was counted as described above.

Using the precise duration of sperm storage as a covariable showed a worse model fit (AIC = 118.02) than using a binary variable (stored/ non stored) (AIC = 109.98) (see Data analysis). Perhaps this is because the exponential decline in sperm numbers produces the largest
variation in sperm numbers early in the storage process (Reinhardt et al. 1999). Storage was, therefore entered as a binary variable (0/1).

**Body size**
Femur length is a good measure of body size in *C. parallelus* (Tregenza et al. 2000), so the mean of the left and right hind femur was measured by a pair of callipers. A different sample of the two populations revealed significant size differences of E (9.08 ± 0.34 mm, N = 25) and P males (9.96 ± 0.45 mm, N = 31) (t = 8.12, df = 54, p = <0.001). In order to prevent masking the population effects I nested femur size within population in the present analysis.

Reinhardt, K. & Meister, J. 2000 Low numbers of sperm retained in the spermatheca may explain the high values of sperm precedence in the migratory locust, *Locusta migratoria* (Latr.). J. Insect Behav. 13, 839-849.