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RESEARCH NOTES

A new technique to assess the number of spermatozoa in spermatophores of stylommatophoran gastropods

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The ultrastructure of molluscan spermatozoa is of systematic significance and has been studied fairly extensively.[1–5] In contrast, little is known in molluscs on the number of spermatozoa transferred to the mating partner during copulation (for exceptions see[6,7]). Many species of gastropods and cephalopods are promiscuous and they have different forms of sperm storage providing the potential for sperm competition.[8] Sperm number is an important determinant in achieving fertilization success in sperm competition.[9] Accurate assessment of the number of sperm transferred to the partner either in form of free sperm (i.e. as sperm suspension in seminal plasma) or sperm encapsulated into spermatophores is an essential prerequisite of any study of sperm competition.

In this note we describe a new technique to assess the number of sperm encapsulated in single spermatophores of stylommatophoran gastropods. Spermatophores of the outcrossing, simultaneously hermaphroditic land snail *Arianta arbustorum* (L.) were used to develop the technique.

Arianta arbustorum is common in moist habitats of north-western and central Europe.[10] Adult snails have a globular shell which measures 17–23 mm in shell breadth. Copulation is reciprocal and includes simultaneous intromission and spermatophore transfer. The spermatophore has a distinct form consisting of a head, a body (sperm container) and a 2–3 cm long tail.[11]

Snails were collected at two sites: (1) in a subalpine forest near Gurnigelbad, Switzerland (46° 45′ N, 7° 28′ E; altitude 1250 m a.s.l.) on 27 April 1996, and (2) on the Ebersangeralm, an alpine meadow in the Ennstaler Alpen, Austria (47° 34′ N, 14° 39′ E; altitude 1500 m a.s.l) on 6 June 1996. The snails were kept isolated in transparent plastic beakers (6.5 cm in diameter, 8 cm deep) lined with moist soil mixed with powdered limestone. Fresh lettuce was provided *ad libitum* as food.

Snails were allowed to mate with a randomly chosen partner. Spermatophores were obtained from snails frozen immediately after copulation. The female reproductive duct of the receiver was dissected to remove the spermatophore. For each spermatophore the length and width of the sperm containing part were measured to the nearest 0.1 mm using a binocular microscope equipped with an ocular micrometer. The volume of the sperm container was calculated assuming a cylindric form. Spermatophores were kept singly in Eppendorf tubes at −40° C until required.

The spermatophore of *A. arbustorum* consists of a hardened secretion which encapsulates the spermatozoa.[11] As in other helicid snails, the spermatophore is attacked by digestive enzymes in the female tract soon after copulation.[12] We mechanically disrupted the spermatophore in 200 ml PBS-buffer (138.6 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4*$2H_2O$ and 1.5 mM KH_2PO_4) using a pair of micro scissors. The sperm suspension was homogenized using a set of Gilson pipettes (in decreasing order 1000 µl, 200 µl and 20 µl pipettes) for 5 to 15 minutes. To obtain openings of different widths, pieces of different lengths were cut from the tips of the pipettes. For a quantitative assessment of the number of sperm, the homogenate was stained with an equal volume of a gallocyanin-chromium complex which stains the DNA in the head of the spermatozoa. This complex is a lake-cation binding optimally at pH 1.6.[13] The staining is independent of its duration and not affected by the presence of ethanol. The sample was stained for 1 to 3 hours and checked for sperm clusters using a light microscope (magnification 400 ×). If spermatozoa still occurred in clusters, the sample was treated with a sonicator (35 kHz) for 16 hours (in cases of extremely dense clusters up to 80 hours). If the spermatozoa were well separated in the sperm suspension, two subsamples of known volume were diluted 1:3 with PBS-buffer and transferred to a Bürker-Türk counting chamber. A counting chamber consists of 16 cells each with a volume of 25 nl. All sperm heads were counted in randomly chosen cells until the total number of sperm heads exceeded 400. Sperm were counted in two subsamples, and the average of these subsamples was used to calculate the total number of sperm in a spermatophore.

In a single subsample, the coefficient of variation of the error of estimate is smaller than 5% when 400 or more spermatozoa are counted, assuming that the number of sperm per cell fits a Poisson distribution (i.e. the spermatozoa are randomly distributed over the cells). This assumption was tested using the number of sperm counted in cells of single spermatophores. In all cases (N = 10 spermatophores)

the number of sperm counted in single cells fitted a Poisson distribution (X^2-test, in all cases $P > 0.5$).

The reliability of multiple sperm counts on the same spermatophore was assessed by calculating the repeatability, i.e. the intraclass correlation coefficient,[14] which is based on variance components derived from an one-way analysis of variance (ANOVA). The repeatability of sperm counts (comparing eight repeated counts from the same spermatophore) was 0.997 ($F_{5,42} = 2939.8$, $P < 0.0001$), indicating a high accuracy of the technique.

The number of spermatozoa was assessed in 35 spermatophores from snails of the Gurnigel population and in six spermatophores from snails of the Ebersangeralm population. Sperm number ranged from 835,000 to 4,974,000 (median: 1,962,700) in the Gurnigel population and from 588,250 to 2,883,200 (median: 1,895,350) in the Ebersangeralm population.

In the Gurnigel population the volume of the sperm container of the spermatophore averaged 2.9 mm^3 (SD = 0.9, range: 1.6–6.4 mm^3, N = 35) and was positively correlated with the sperm number ($r = 0.766$, $N = 35$, $P < 0.0001$).

The technique described in this note gives an accurate estimate of the number of sperm transferred in single spermatophores and thus provides the opportunity to study various aspects of sperm competition in this simultaneous hermaphrodite.[15,16] The technique might also easily be adjusted to spermatophores of other stylommatophoran species (e.g. *Helix aspersa* and *Cepaea nemoralis*) and possibly for other invertebrates which use spermatophores for sperm transfer.

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The first record of 'accidental' copulation between male squid of the genus *Illex*

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Ommastrephid squid represent about 90% of the total squid catch world-wide making them an important fisheries resource.[1] Three ommastrephid squid species *Illex coindetii* (Verany, 1839), *Todaropsis eblanae* (Ball, 1841) and *Todarodes sagittatus* (Lamarck, 1798) occur in Irish waters. The fisheries biology and life cycle of two of these, *I. coindetii* and *T. eblanae* has been under investigation for the past three years[2] and as part of this study all the squid caught during MAFF's (Ministry of Agriculture, Fisheries and Food U.K.), Celtic Sea Groundfish surveys in March 1994 and 1995, were examined in detail.

During the survey in March 1995, a total of 384 *I.*