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Stereological analysis of the sperm number in the testicular follicles of the Australian field cricket (Insecta: Orthoptera)

Robert STURM

A b s t r a c t : Recent studies have proposed the hypothesis that each spermatophore of the male Australian field cricket is supplied with the sperm cells stored in a single testicular follicle. In order to confirm or reject this theory, spermatozoa developed in the follicles of mature males (age: > 5 days) have been counted with the help of stereological techniques. For an unbiased estimate of the total cell number in the structure of interest a combination of the Cavalieri principle and the optical disector method was applied, thereby using a series of optical sections (separated by 2 µm) of the male reproductive organ. According to this counting procedure the number of spermatozoa stored in a single follicle amounts to 92,771 ± 42,138 cells. This result clearly speaks in favour of the hypothesis stated at the beginning.

K e y w o r d s : Stereology, spermatozoa, testicle, *Teleogryllus commodus*, Orthoptera.

Introduction

As already outlined in several previous studies, the testicles in males of the Australian field cricket (*Teleogryllus commodus* WALKER, 1869) are characterized by their abdominal position above the gastrointestinal tract, where they cling to a median plane running through the insect body (STURM 2003a, 2003b, 2014a). The reproductive organs commonly contain a specific number of tubular follicles, where development of the male germ cells from initial spermatogonia to fully differentiated spermatozoa takes place (spermatogenesis; WEBER & WEIDNER 1974, STURM 2014a). In the case of *Teleogryllus*, the number of testicular follicles varies between 80 and 120 and thus ranks in an order of magnitude being rather typical for Orthopteran insects (SPANN 1934, WIGGLESWORTH 1972, CHAPMAN 1998, HALL et al. 2000; Fig. 1, 2).

The main task of the testicles consists in the supply of spermatophores with germ cells. Thereby, the transport of the spermatozoa takes place over the ductus ejaculatorius, and a temporary storage of the cellular material is enabled through the seminal vesicles (STURM 2015).The fully developed spermatophore is afterwards transferred to the female in such way that its tube is threaded in the ductus receptaculis and the sperm can be pumped from the ampulla to the female spermatheca (STURM 2002a, 2003a, 2003b, 2008a, 2012). As already found by KHALIFA (1949) completed spermatophore of cricket species are marked by a very regular arrangement of the spermatozoa with parallel alignment of the cell bodies and flagella. In the case of the Australian field cricket this specificity was used to evaluate the number of sperm stored in the ampulla by application of different microscopic techniques (STURM 2011, 2014b). As could be demonstrated by comprehensive analyses, the amount of spermatozoa varies somewhere

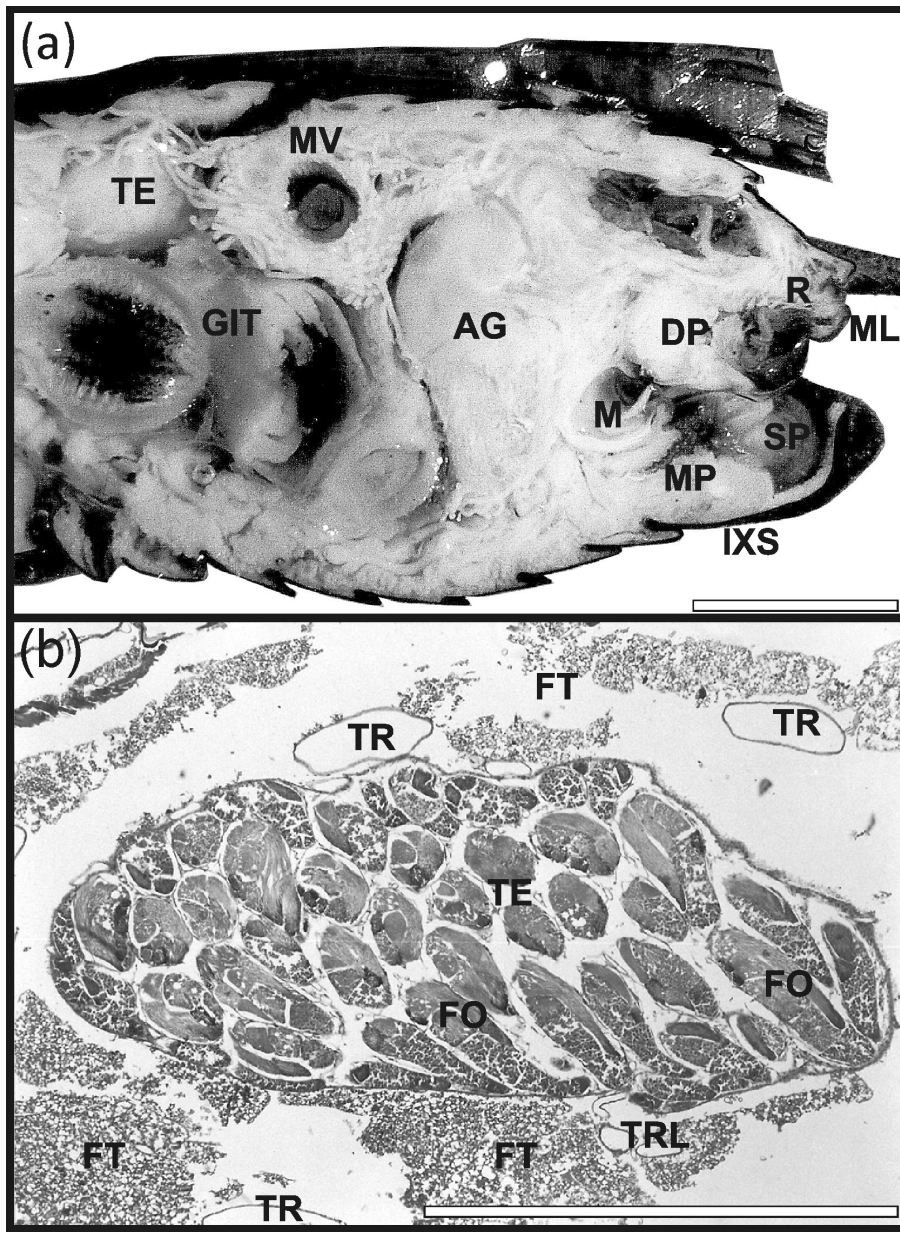

Fig. 1: (**a**) Stereo-microscopic image of a median section through the male abdomen of the Australian field cricket with its main organic structures; (**b**) Light-microscopic image of the testicle with follicles representing the places of spermatogenesis. Abbreviations: AG - accessory glands, DP - dorsal pouch, FO - follicle, FT - fatty tissue, GIT - gastrointestinal tract, M - mould, ML - median lobe, MP - median pouch, MV - Malpighi vessels, R - rectum, TE - testicle, TR - trachea, TRL - tracheole, IXS - 9th segment.

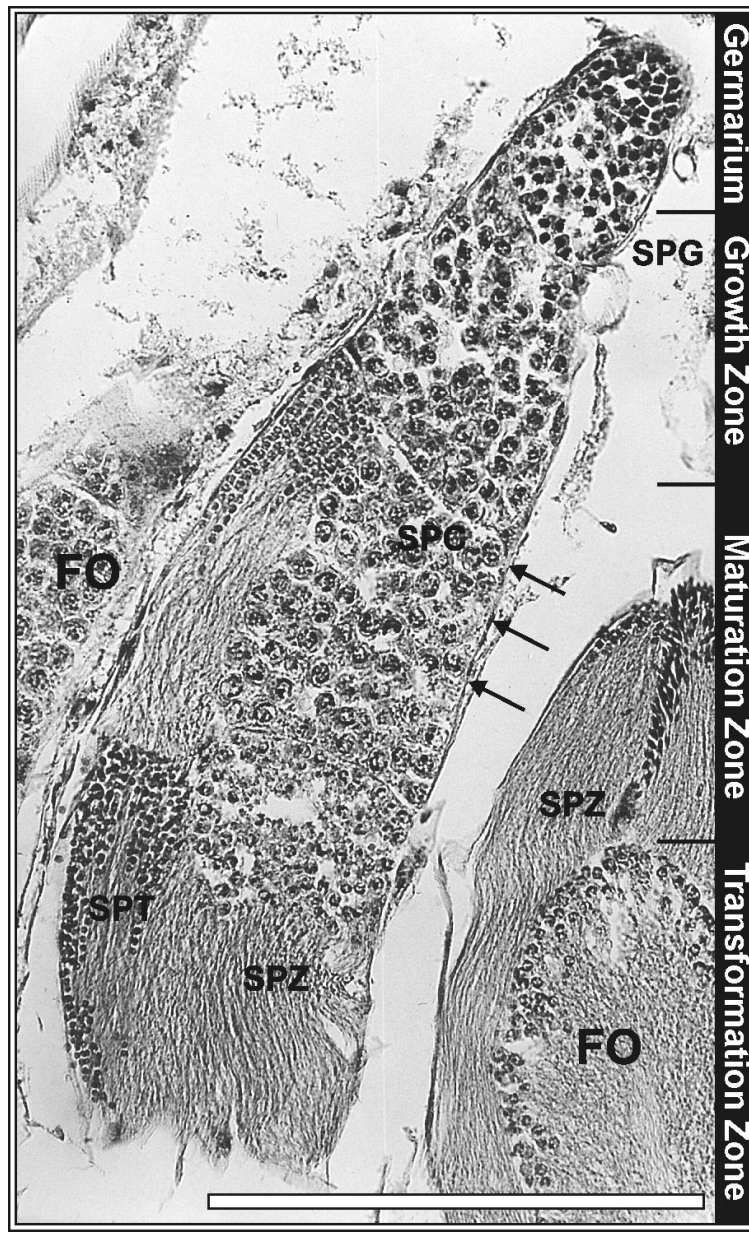

Fig. 2: Detailed light-microscopic photograph of a testicular follicle with its different zones of sperm development (right margin). For sperm counting only the basal part of the transformation zone was used. Abbreviations: FO - follicle, SPC - spermatocytes, SPG - spermatogonia, SPT - spermatids, SPZ - spermatozoa.

between 90,000 and 150,000 cells and among other depends on the size and, thus, physical fitness of the male.

In a previous contribution (STURM 2003b) it was hypothesized that the number of spermatozoa stored in a single spermatophore should correspond with the amount of sperm cells formed in a testicular follicle. This would mean that one single follicle assumes responsibility for the total supply of a sperm container with germ cells. In the present contribution the number of fully differentiated spermatozoa within the testicular structures is evaluated with the help of stereological methods and compared with the published amount of sperm cells collected in the ampulla.

Material and Methods

For the production of optical sections through the testicular structures selected males (N = 10) were anaesthetized in a CO_2 stream and afterwards decapitated. The isolated testicles were dehydrated in an ascending alcohol series (70-100% ethanol) and fixed in classical Bouin solution, which is composed of picric acid, acetic acid, and formaldehyde in an aqueous solvent. Production of oriented sections through the reproductive organs was carried out by embedding of the rather soft structures in a specific mixture of epoxy resin allowing their optimal treatment. The sections were applied on glass slides (76 x 26 mm) and freed from the embedding medium representing a disturbing factor during light-microscopy. After staining of the preparations according to the Goldner or Azan procedure, the sections were finally provided with Canada balsam (n = 1.53) and a cover slip (STURM 1999, 2002a, 2002b, 2002c, 2008b, 2017, STURM & POHLHAMMER 2000).

For the counting of spermatozoa in the optical sections the stereological method outlined by BERTRAM & NURCOMBE (1992) was used. This procedure has the advantage that it does not require assumptions or knowledge of cellular shape or size and hence provides unbiased estimates. The so-called optical disector-Cavalieri combination connects the stereological advantages of the disector with the physical laws of optics. As depicted in Fig. 3, cell bodies are quantified in a specific counting frame at their sharpest focus. The counting frame is either placed in the eyepiece lens or inserted in the projection lens if the image is to be viewed on a monitor. The counting procedure is illustrated in detail in Fig. 3: Whilst cell bodies touching the bold lines of the frame are included in the quantification, those cells touching the dashed lines are excluded from the process. The area of the counting frame multiplied by the depth of the section used for counting gives the analyzed volume. The numerical density of cells (number per unit volume $N_{Vcell,ref}$) can now be calculated.

For estimating the volume of the reference space the Cavalieri principle is used. For this purpose the combined area (A) of a known fraction (f) of sections is computed. Now, the volume of the reference space (V_{ref}) can be derived from the equation

$$V_{ref} = A(1/f)t, \quad (1)$$

where 1/f represents the inverse of the section sampling fraction and t the section thickness (2 µm). An unbiased estimate of the total cell number (N_{cell}) is obtained by simply multiplying cell numerical density ($N_{Vcell,ref}$) by Volume of the reference space (V_{ref}):

$$N_{cell} = N_{Vcell,ref} \times V_{ref}. \quad (2)$$

As proposed by BERTRAM & NURCOMBE (1992), the method described above is well suited to studies of small structures, where V_{ref} and $N_{Vcell,ref}$ can be estimated in the same microscopic sections. In this case, the effects of shrinkage/swelling artifacts on volumes are effectively eliminated.

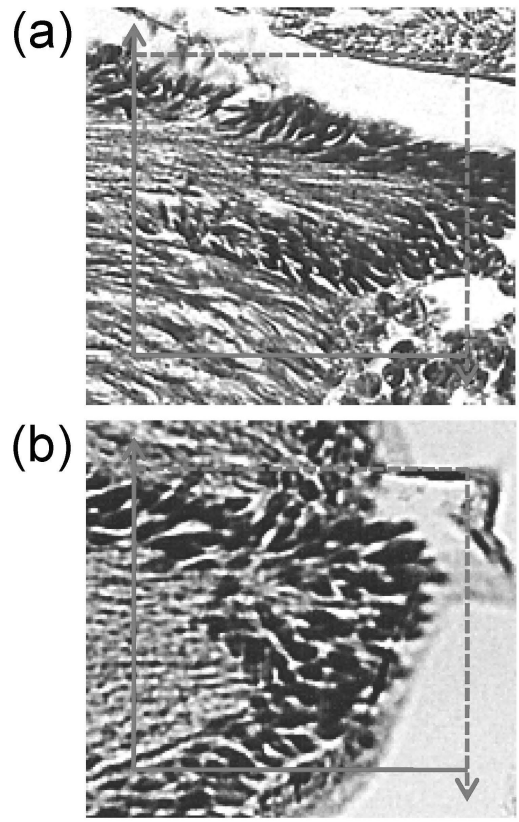

Fig. 3: Stereological quantification of spermatozoa by using a combination of the Cavalieri principle and disector method. The reference frame (20 x 20 µm) plays an important role insofar as cells touching the fully drawn line are included in the counting procedure, whereas cells touching the dashed line are excluded.

Results

As already mentioned in the methods section, a total of ten males (age > 5 days) was included in the stereological investigation. From each animal ten follicles were investigated for their contents of fully differentiated spermatozoa. Reference volumes (V_{ref}) of the analyzed structures varied between 0.3 x 10^{-5} mm³ (3,000 µm³) and 2.3 x 10^{-5} mm³ (23,000 µm³). Number of cells per unit volume ($N_{Vcell,ref}$) ranged from 11 to 76. Respective results of the counting procedure are summarized in Tab. 1.

Tab. 1: Results of the stereological counting procedure ($T_{1\text{-}10}$ = testicle 1-10, $F_{1\text{-}10}$ = follicle 1-10, m = mean value, sd = standard deviation).

	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
F_1	143386	46180	135506	32624	155201	145092	147651	95748	134662	81807
F_2	127094	158567	150815	125825	62249	136150	76598	112060	113423	72597
F_3	72668	61181	90026	68200	24347	47778	96615	116464	105083	91719
F_4	125980	124081	114965	35859	117077	149192	20457	74468	106052	155905
F_5	107057	25461	68668	132853	26556	42001	79887	26338	60510	71883
F_6	150202	146373	95172	109464	20510	61495	91598	98662	93165	56984
F_7	67911	95481	132429	81677	36195	108750	95023	**11965**	140086	21065
F_8	67176	68274	132115	94451	39416	**159821**	114176	116582	143597	13393
F_9	40301	158642	119255	42418	145471	99062	13732	101297	67704	75895
F_{10}	54657	73931	121838	27816	131048	155411	103683	123499	117147	148517
m	**95643**	**95817**	**116079**	**75119**	**75807**	**110475**	**83942**	**87708**	**108143**	**78977**
sd	**39645**	**48408**	**24758**	**39725**	**54894**	**45902**	**40462**	**38848**	**28347**	**46144**

As can be clearly recognized from the above inserted table, number of spermatozoa varies remarkably from follicle to follicle and from animal to animal. Minimal amount of sperm cells (11,965) could be detected for follicle 7 of animal 8, whereas maximal amount of sperm cells (159,821) was determined for follicle 8 of animal 6. This, however, results in a range of 147,856 cells. By application of basic statistics mean values and related standard deviations of the estimates listed in Tab. 1 were computed. These statistical parameters range from 75,119 $\pm$ 39,725 cells to 116,079 $\pm$ 24,758 cells. Mean value and standard deviation calculated over all follicles and animals amount to 92,771 $\pm$ 42,138 spermatozoa.

Discussion and Conclusions

Stereological counting methods have become a primary feature of biological and medical sciences during the past decades. With the help of specific quantification techniques an unbiased estimate of cell numbers within a given reference volume is possible (BERTRAM & NURCOMBE 1992, STURM 2002a). Stereoscopic techniques are ideally suited to any kinds of light-microscopic investigations, whereas there is a difficulty in applying them in electron-microscopy. This can be mainly explained by the fact that respective sections used for transmission electron-microscopy measure several nanometers in thickness and thus need to be produced in high numbers to obtain a representative picture of cell distribution within an organ. The optical disector-Cavalieri combination used in this study enables the analysis of a very limited number of optical sections, so that only 100-200 cells need to be counted per analyzed structure. On the other hand, all extrapolations summarized in equations (1) and (2) presuppose a homogeneous distribution of the cells through the tissue, which was not perfectly realized for the testicles of *Teleogryllus*. Therefore, respective inaccuracies of the final estimates had to be accepted.

Statistical combination of all estimates presented in Tab. 1 resulted in an overall sperm number of 92,771 ± 42,138 cells per follicle. As found by STURM (2002a, 2003a, 2011, 2014b) the amount of spermatozoa provided for a single spermatophore is on der order of 152,000 ± 32,000 cells and therefore does not deviate significantly from the numerical results presented in this contribution. This interesting circumstance, however, gives rise to a confirmation of the hypothesis outlined by STURM (2003a, 2003b), according to which filling of a spermatophore is mainly conducted by a single testicular follicle. This means that the male of *Teleogryllus* 'empties' one follicle after the other in order to guarantee high repetition of spermatophore transfer within a rather short period of time. Regeneration of the full number of spermatozoa per follicle seems to be much more time-consuming than production of spermatophores (STURM 2011), so that this strategy of sperm transfer from the reproductive organ to the transport device could indeed make some sense.

It has to be concluded with emphasis that the findings of this paper may not be evaluated as strong evidence for the hypothesis stated above, but show a tendency towards this theory. In order to obtain a better and more profound relationship between quantities involved in sperm production and sperm transfer, further highly targeted studies have to be carried out.

Zusammenfassung

Stereologische Analyse der Spermienzahl in den Hodenfollikeln der australischen Feldgrille (Insecta: Orthoptera). – Neuere Studien vertreten die Hypothese, dass jede Spermatophore der australischen Feldgrille mit den in einem einzelnen Hodenfollikel gespeicherten Samenzellen versorgt wird. Zur Bestätigung oder Verwerfung dieser Theorie wurden die in den Follikeln von reifen Männchen (Alter: > 5 Tage) gebildeten Spermatozoen mithilfe von stereologischen Techniken quantifiziert. Für eine erwartungstreue Schätzung der Gesamtzellzahl in der interessierenden Struktur gelangte eine Kombination aus Cavalieri-Prinzip und optischer Dissektor-Methode zur Anwendung, wobei eine Serie von Gewebeschnitten (Abstand: 2 µm) durch das männliche Reproduktionsorgan verwendet wurde. Gemäß dieser Zählprozedur beläuft sich die Anzahl der in einem einzelnen Follikel gespeicherten Spermatozoen auf 92.771 ± 42.138 Zellen. Dieses Ergebnis spricht recht klar für die eingangs erwähnte Hypothese.

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Anschrift des Verfassers: MMMMMMag. Dr. Robert STURM
Brunnleitenweg 41
A-5061 Elsbethen, Austria
E-mail: sturm_rob@hotmail.com