Charles University in Prague
Faculty of Science
Department of Zoology

Cytogenetics and biology of selected representatives of the family Sphaeriidae

Cytogenetika a biologie vybraných zástupců čeledi Sphaeriidae

PhD. thesis

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Prague, January 2011

Declaration

I hereby declare that I did not previously submit this Ph.D. thesis or its part to fulfil conditions of the same or of another university degree. The results presented here have been obtained in the course of my research work and consulted with my supervisor. The level of participation (by help or advice) of other persons is further specified in Acknowledgements and in the particular articles.

Prague 3rd January 2011 Tereza Kořínková

Prohlášení

Prohlašuji, že jsem tuto práci ani její podstatnou část nepředložila k získaní jiného nebo stejného akademického titulu. Výsledky zde prezentované jsem získala samostatnou prací, průběžně konzultovanou se školitelkou a s přispěním rad a pomoci osob, jejichž podíl je specifikován v kapitole “Ackowledgements” a u jednotlivých předkládaných článků.

V Praze 3.1. 2011 Tereza Kořínková

Contents

Motto:

"Da steh ich nun, ich Armer Tor, und bin so klug wie zuvor".

(Goethe: Faust)

Dedication

I would like this thesis to be in memory of Alexei V. Korniushin (1962-2004) – a phenomenal expert in the problematics of Sphaeriidae. It is fascinating what a piece of work one man was able to do in his, unfortunately precociously finished, life.

Acknowledgements

I had the opportunity to use diverse techniques and approaches in the course of this work. As nobody is an expert in all fields, I sometimes needed a help or advice of other persons.

First of all I would like to express my thanks to my supervisor Lucie Juřičková, who has given me the most of the possible freedom to plan the process of my work. She has, together with Michal Horsák (Department of Botany and Zoology, Masaryk University Brno) and Luboš Beran (Kokořínsko PLA Administration), inspired me to choose Sphaeriidae as an object of my study, and these three persons were also most active in helping me to obtain suitable material. They informed on suitable localities or accompanied me to some field trips, or even provided me with material from hardly accessible sites. I must also appreciate other colleagues that have helped me in the same way – Heike (and Benjamin) Reise (Senckenberg Museum für Naturkunde Görlitz), Romualda Petkevičiūtė, Virmantas Stunžėnas and Gražina Stanevičiūtė (Institute of Ecology, Vilnius University) and Bartłomej Gołdyn (Department of General Zoology, Adam Mickiewicz University, Poznan). The colleagues from Vilnius University and Taehwan Lee from Michigan have moreover contributed valuable advice concerning chromosome preparation. B. Gołdyn was also my kind host at the Department of General Zoology, where I was enabled to produce some of the chromosome preparations of *Pisidium* (see Chapter 1). Most of the karyotype data have been obtained in the Laboratory of Arachnid Cytogenetics (Department of Genetics and Microbiology, Faculty of Science, Charles University Prague) under supervision of Jiří Král or at the Department of Zoology of the same faculty using the facilities kindly provided by František Šťáhlavský. To J. Král I owe my immense thanks, as he, without being my official supervisor, has first spent a lot of his precious time introducing me into the techniques of chromosome preparation and later, as my boss, let me use in my free time the facilities of his laboratory for evaluation of my own material. As an experienced cytogeneticist, he has helped me to interpret the obtained results, which make up a substantial part of my thesis. Therefore, he is by right a co-author of the publication on B chromosomes (Chapter 2).

Measurement of DNA contents by flow cytometry, though not included in the original project, has contributed valuable data, which could not be obtained without the indulgent and patient work of A. Morávková, who is a co-author of the publication concerning polyploidy in Sphaeriidae (Chapter 1).

Histological sections, used for life-history studies (Chapter 3), could not be prepared without advice given by Milada Řeháková. By retirement of this experienced co-worker, the Department of Zoology has lost not only an excellent technician, but also one of persons that were making the place nice and homely.

Preface

Whenever explaining to non-scientists the rationale of my thesis, I am asked the question „what is it good for?" „What sense does it have?" „What practical applications will it yield?"

As if exploring live organisms, their diversity and function always had to be of practical importance, as if it had no sense per se. Many famous discoveries that have lead to practical applications have been made unintentionally in course of „basic research". And on the other hand, exciting pieces of information on the diversity of life are being acquired as „by-products" of applied research.

The object of my study, Sphaeriidae (fingernail-, pea- or pill-clams) belong to a globally important clade of primarily freshwater bivalves and make a substantial part of numerous freshwater ecosystems. As such, they even have a „practical importance" e.g. for fishery, water management, as indicators in water ecology or as intermediate hosts of parasites. One should also take into account other aspects, as their reproductive strategy (viviparity) and cytogenetical peculiarities (paleopolyploidy, occurrence of B-chromosomes), which make sphaeriids perspective model organisms. No wonder that this group of organisms is in the last decade being intensely investigated with respect to all these aspects.

My thesis aimed on cytogenetics and selected aspects (life-histories, food intake) of Sphaeriidae biology. The obtained results might be of importance for other disciplines, e.g. for taxonomy (the main taxonomical problems are briefly mentioned in the Introduction and Conclusion section). The data should contribute to the mosaic of recent knowledge on the topic and represent a basis for further investigations. It might iniciate further applied research, or remain just a piece of „blue-sky science".

Introduction

Sphaeriidae are a family of primarily freshwater bivalves. Their appearance is featured by a rounded (oval, spherical – therefore the names „*Sphaerium*“, „*Pisidium*“) shell outline with more or less prominent umbones (see Figure 1 for explanation of the terms) and with a heterodont dentition (hinge composed of morphologically differentiated anterior, posterior and cardinal teeth). Many sphaeriid species belong to the smallest bivalves – the largest dimension of the shell (the shell length) ranges in adults from ca 1-2 mm (*Pisidium moitessieranum*) to 25 mm (*Sphaerium rivicola*).

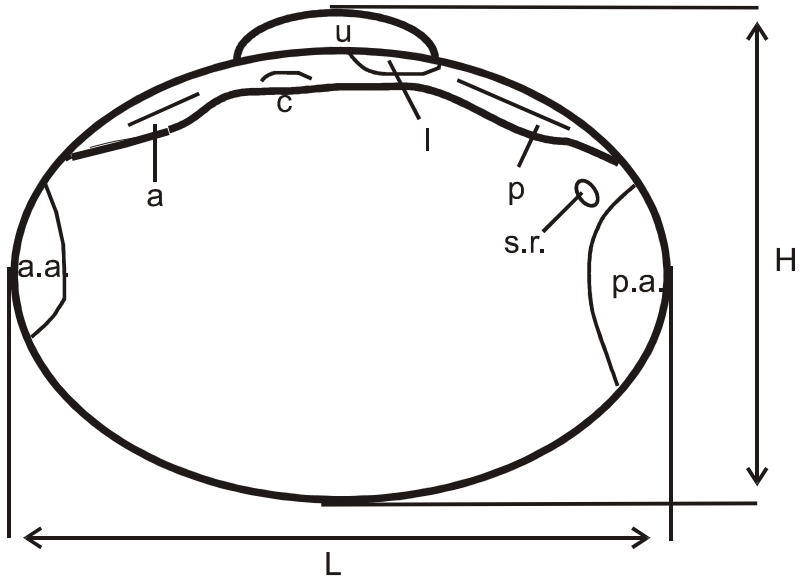

Figure 1 – Schematic drawing of *Sphaerium* shell (right valve, lateral view from inside) with explanation of the terms: a – anterior teeth, a.a. – scar of anterior adductor, c – cardinal tooth, l – ligament, p – posterior teeth, p.a. – scar of posterior adductor, s.r. – scar of a siphonal retractor muscle, u – umbo, H – shell height, L – shell length

The recent distribution comprises almost all zoogeographic regions except Palaearctic and Nearctic. The first reliable fossil record is known from Cretaceous (Keen and Dance 1969), the shells from Pleistocene and Holocene are often well-preserved including the organic layer and they correspond to those of the recent species (Nylander 1909 rev. in Martin 1998, Kuiper 2009).

Taxonomy

Although this thesis touches the taxonomical problematics only briefly, a short outline of the main problems is necessary for understanding the relations between the studied species.

The actual number of valid sphaeriid species cannot be reliably evaluated due to often imprecise descriptions of the species and inconsistencies between concurrent taxonomic

schools. For example, the first described representative of the family was *Sphaerium corneum* (Linné 1758). Interestingly, the population on which this description has been based, probably did not belong to *S. corneum* in the present sense. As noted by Falkner (2000), the description rather complies with characters typical for *S. nucleus* (Studer 1820) sensu Korniushin (2001). As Studer´s (1820) description was quite vague (actually just three lines briefly describing the shell outline), many authors did not accept *S. nucleus* as a valid species and regarded it as a subspecies or „form" of *S. corneum*. To enhance the confusion – there is no type material for any of the two original descriptions. Evidences given by many authors show the existence of at least two distinct, though conchologically hardly distinguishable *Sphaerium* species in Central Europe (Falkner 2000, Mildner 2001, Kořínková 2006a). These could be assigned the names *S. corneum* and *S. nucleus*. According to Korniushin (2001), they belong to a group of closely related species ("*S. corneum* group") together with *S. radiatum*, which has also been regarded as a conchological form of *S. corneum* by some authors and as a separate species by others. According to Korniushin (2001), *S. radiatum* is a valid species, with a synonym *S. ovale*. Nonetheless, some representatives of the so-called „Russian taxonomical school" distinguish even more Palaearctic species of the „*S. corneum* group". A similar situation occurs also in the other sphaeriid genera – the original descriptions are based on few shell characters and do often not comply with the biological species concept.

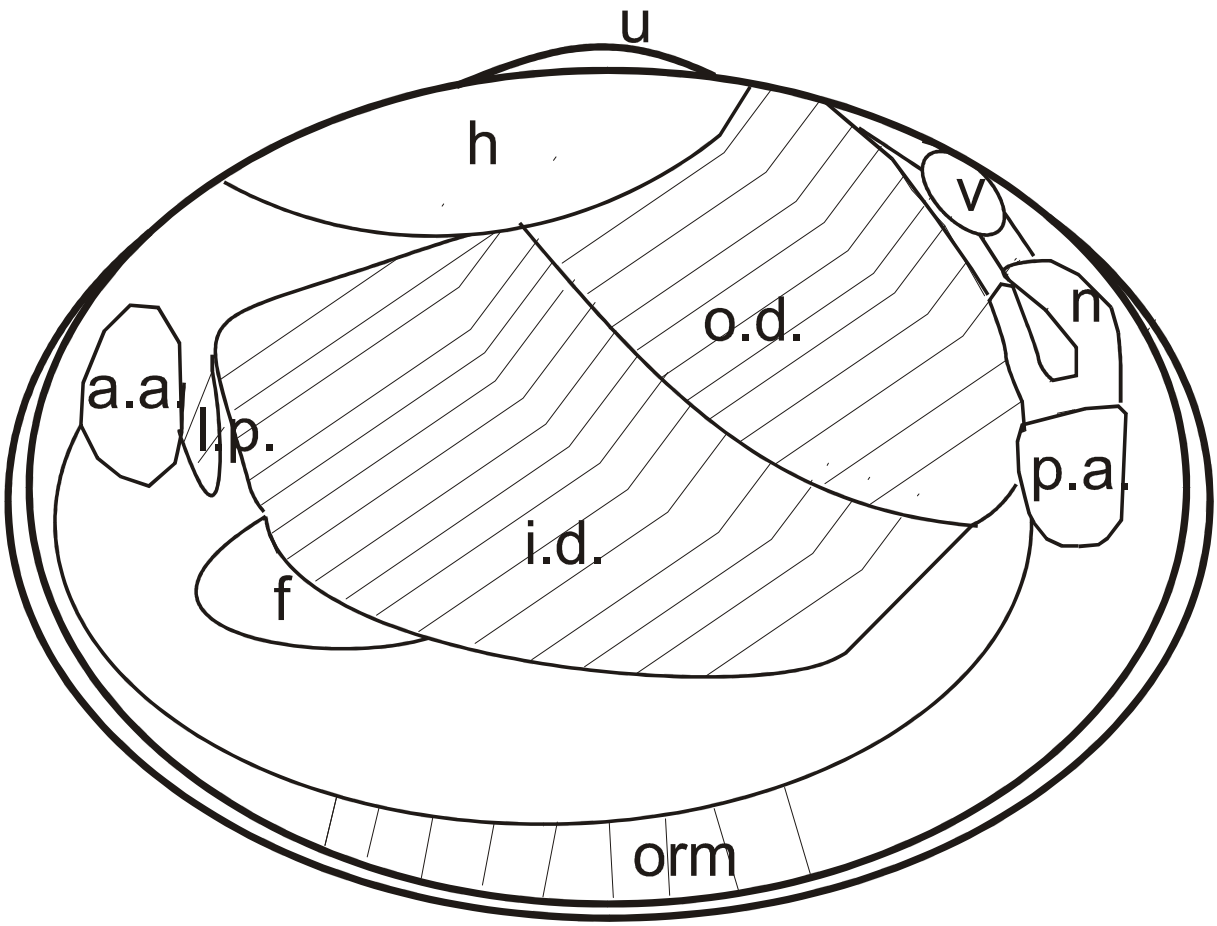

Figure 2 – general anatomy of a sphaeriid clam – left lateral view after removal of the left valve and the mantle. a.a. – anterior adductor, f – foot, h – hepatopancreas, i – intestine, i.d. – inner demibranch, l.p. – labial palp, orm – outer retractor muscles of mantle, n – dorsal lobe of nephridium, p.a. – scar of posterior adductor, u – umbo, v – ventriculus.

New sphaeriid species are still being described, especially from tropical regions (see e.g. Ituarte 2004). The new descriptions usually combine conchological characters with those based on internal anatomy, like the shape of dorsal kidney lobe, arrangement of retractor muscles etc. (see Figure 2 for explanation of the anatomical features).

All species studied within the frame of this thesis have Palaearctic distribution. The *Pisidium* species were determined using shell characters, whether the distinction of the problematic sibling species *S. corneum* and *S. nucleus* was based mainly on previously published anatomical characters (Korniushin 2001, Kořínková 2006a).

Further paragraphs represent a brief overview of previous knowledge on the aspects investigated in this thesis (i.e. cytogenetics, life-history and food intake).

Cytogenetics of Sphaeriidae

Whereas the conchological variability and reproductive biology of sphaeriids has been widely studied since the 19th century, the first study on cytogenetics of a fingernail clam comes from the half of the 20th century. Although some stages of meiotic and mitotic division have been observed (usually with unsatisfactory results) even on paraffin sections (Woods 1931), the first cytogenetic study comes from Keyl (1956). He has given a detailed and, as will be shown later (Chapter 2), quite accurate description of the meiotic division in a population of *Sphaerium*.

The first reports on chromosome numbers in the genus *Pisidium* appeared in 1960s and 1970s (Burch and Huber 1966, Burch 1975). All the hitherto studied *Pisidium* species and most species of *Sphaerium* and *Musculium* exhibited high chromosome numbers (150-247) (Burch and Huber 1966, Baršienė et al. 1996, Burch et al. 1998, Lee 1999, Lee and Ó Foighil 2002, Park et al. 2002, Jara-Seguel et al. 2005, Petkevičiūtė et al. 2007).

Lee (2001) has proven by sequencing of single-copy genes that most North American sphaeriids possess multiple alleles exhibiting cross-species sister relationships, suggesting a previous reticulate speciation by allopolyploidization. It has been shown that polyploidization has played an important role in the speciation of organisms (Otto and Whitton 2000). The genomes of ancient polyploids (paleopolyploids) have been stabilized by the process of diploidization, involving structural rearrangements of chromosomes and partial elimination of the genome (Wendel 2000, Wolfe 2001, Ma and Gustafson 2005). In plants, up to 80% of species are considered paleopolyploids (Otto and Whitton 2000). In animals, polyploidy usually causes low viability and fecundity, especially in species with sex chromosome determination (Müller 1925) by disruption of sex determination mechanisms or altered dosage of sex-linked genes. In effect, evolutionary significance of polyploidy in animal evolution has been underestimated in the past (Otto and Whitton 2000). However, some animals (usually asexual, hermaphroditic or parthenogenetic) are able to cope with, or even to profit from, polyploidy (Comai 2005) and the polyploid genome is maintained. The reason why polyploid lineages often reproduce asexually is the reduced capability of gamete production due to the irregularities in meiotic pairing and segregation. In autopolyploids, the presence of more than two homological copies of each chromosome often causes formation of multivalents. In allopolyploids, both homologous (between chromosomes of the same parental set) of homeologous (between similar chromosomes of different sets) pairing may occur at least at early prophase of the first meiotic division. However, ancient allopolyploids possess mechanisms that facilitate homologous and hamper homeologous pairing (Martinez-Perez et al. 2003). During the process of diploidization, the risk of homeologue pairing is rapidly eliminated. Therefore, ancient polyploids usually exhibit regular meiosis with bivalent formation and normal gamete formation and sexual reproduction. In Sphaeriidae (and the related families Lasaeidae and Corbiculidae) all hitherto examined species are hermaphroditic,

which is an important prerequisite for polyploidy. Whereas polyploidy in Lasaeidae and Corbiculidae is probably of a recent origin and occurres in connection with andro-, gyno- and parthenogenesis (Ó Foighil and Thiriot-Quievréux 1999, Skuza et al. 2009), in Sphaeriidae no case of asexual reproduction modes has been reported till now. Lee (2001) has observed at first meiotic division of *S. rhomboideum* elements, probably bivalents, the number of which corresponded to half of the chromosome number in somatic cells. Unfortunately, the morphology of the putative bivalents was not clear, but the observation would imply a regular course of meiosis, without formation of multivalents. As only few *Sphaerium* species and no *Pisidium* species have so far been reported to have lower numbers of chromosomes, it was very feasible to investigate other representatives with respect to chromosome number, morphology and meiotic behaviour. This was the aim of Chapter 1, which attempted to answer the following questions:

Do all Central European species of the genera *Sphaerium*, *Pisidium* and *Musculium* possess high chromosome numbers?

If so, do the chromosomes exhibit regular bivalent formation and segregation at meiosis, or is there some tendency towards formation of multivalents?

Are the high chromosome numbers in some species correlated with large genome sizes, which would imply a recent polyploidization? Or have the genomes rather undergone the process of genome size reduction, which is typical for palaeopolyploids?

Lower chromosome numbers have only been found in *S. corneum* (Keyl 1956, Baršienė and Baršytė 2000, Petkevičiūtė et al. 2006), *S. nitidum* (Baršienė and Baršytė 2000) and *S. rhomboideum* (Petkevičiūtė et al. 2007). The material studied by Keyl (1956) was assigned by him as *S. corneum* according to the contemporary opinions on taxonomy of Sphaeriidae. Nevertheless, it can not be ruled out that the population in fact belonged to the sibling species *S. nucleus*. A similar uncertainty exists about the species identity of some *Sphaerium* populations studied by Petkevičiūtė et al. (2006). These authors have reported the presence of supernumerary chromosomes and in one population, co-occurrence of specimens with two distinct karyotypes has been found. Preliminary results obtained for a diploma thesis (Kořínková 2006b) have also indicated a diploid number of 30-36 chromosomes in Czech populations of *S. corneum* and its sibling *S.nucleus*.

This has raised several questions:

What are the real basic chromosome sets of *S. corneum* and *S. nucleus*?

How important is the interpopulational variability?

Are there any significant interspecific differences?

Is the previously found variation of chromosome numbers in the Czech populations caused by the presence of B chromosomes or rather by aneuploidy?

I tried to answer these questions in the Chapter 2.

Structure of the reproductive system, breeding strategies

Another aspect that has been exciting for investigators not less than the uncertain sphaeriid taxonomy and high chromosome numbers is the biology of this bivalvian group. Sphaeriids are benthic filter- feeders or suspension-feeders, often occurring in large population densities. All of them possess a viviparous mode of reproduction. It has been noticed already by Stepanoff (1865), that the larval development occurs within the gills of the parental organism (Figures 3, 4) and young clams are only released as fully-developed, self-sustaining individuals. Since then, many authors have studied details of viviparity on histological sections. Poyarkoff (1910) and recently Hetzel (1993) have described the structure of special protective and nutritive tissues in the gills of the parental organism and provided data on

anatomy and morphology of the respective larval stages incubated within these structures. Beekey et al. (2000) have shown by a laboratory experiment the possibility of precocious release of underdeveloped young clams and their ability to survive. Most studies since the 19th century until present have been dealing with populational biology and life-history aspects connected with viviparity, e.g. the reproductive periods, brood size, longevity, survivor of newborn clams etc. Some papers on the topic are reviewed in Chapter 3, which aimed to analyse in detail the life-history of one *S. corneum* population and to answer the following questions:

Is the timing of gametogenesis, probable fertilization, release of the F1 generation and death of old adult individuals highly synochronized in the population, or are there some individuals exhibiting a different pattern?

How long do the individuals live, what is the age at the onset of reproduction and what size classes of the population do overwinter?

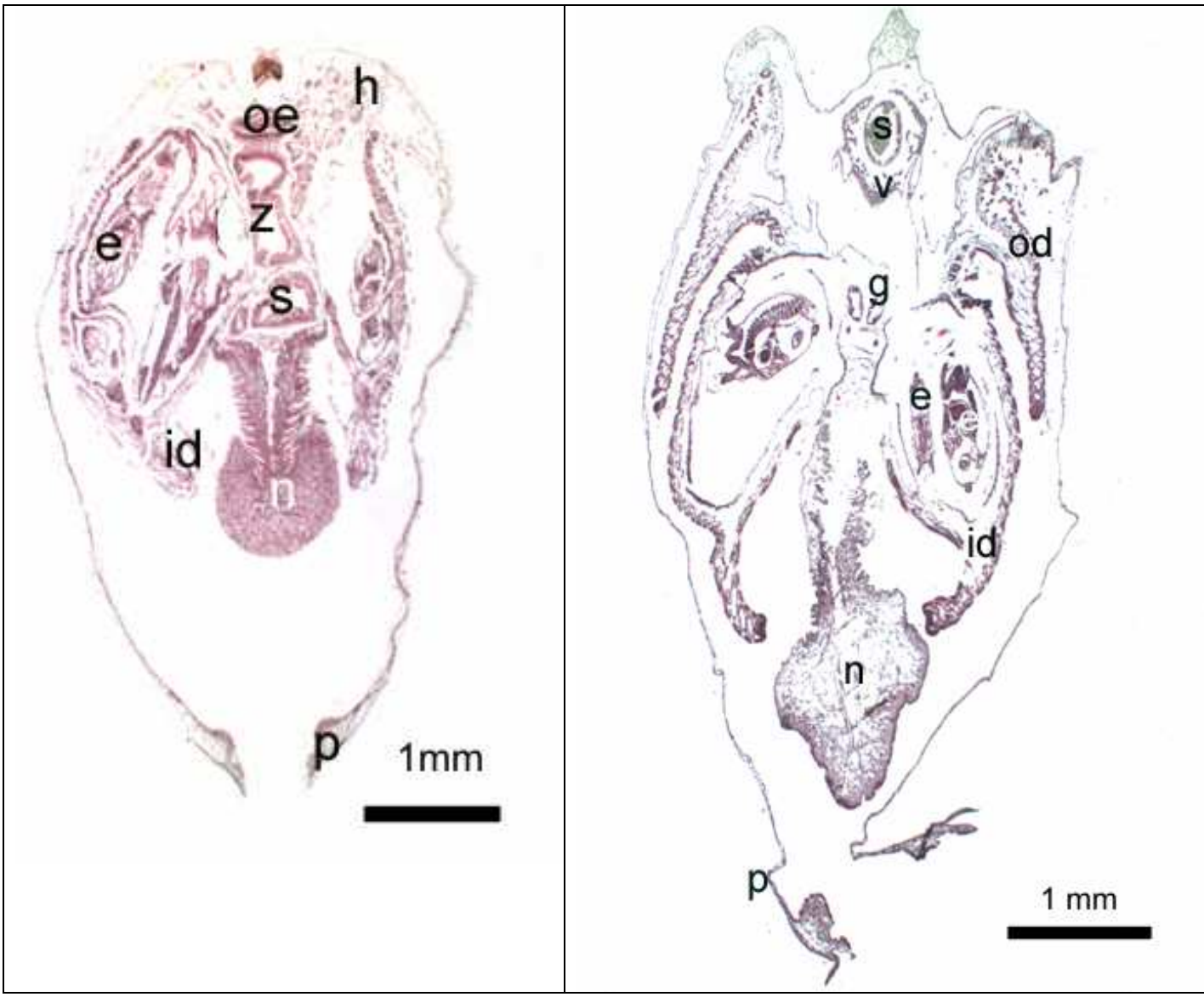

Figure 3 (left) and 4 (right) – anatomy of *Sphaerium*. Paraffin frontal section (5 um) through anterior (Figure 3) and posterior (Figure 4) part of *S. nucleus* body.

e – embryo, h – hepatopancreas, g – gonad, id – gill – inner demibranch, n – foot, -od – gill – outer demibranch, oe – esophagus, p – mantle, s – intestine, v – hearth ventriculus, z – stomach. From Kořínková (2007) with a kind permission of the publisher.

The gross anatomy and microanatomy of breeding structures have been studied already in the diploma thesis (Kořínková 2006b) and presented to the non-scientific public in a popular article (Kořínková 2007). The study presented here ties together with the previous investigations.

Structure and function of alimentary tract

Apart from some peculiarities (like the brooding space in the inner demibranch), Sphaeriidae exhibit a typical eulamellibranchiate anatomy. Numerous experimental studies on non-sphaeriid bivalves have previously dealt with the mechanism of filter-feeding, that involves trapping and sorting of suspended particles using the gills (described in more detail in the Chapter 4), and they have brought data on physical, biomechanical and biochemical parameters like the filtration rate and efficacy (water clearance), mechanical sorting of the particles on the gills and in the stomach and the abundance of digestive enzymes (see e.g. Reid 1968, Navarro et al. 1996, Charles and Newell 1997, Hawkins et al. 1998, Wong and Cheung 2001). However, sphaeriid clams can rather be characterized as interstitial suspension-feeders or deposit-feeders rather than filter-feeders (Mitropolskii 1966, Hornbach et al. 1984, Lopez and Holopainen 1987, Way 1989, Raikow and Hamilton 2001). Only few studies have also reported, usually on the basis of breeding experiments, concrete microorganisms that were utilised by the bivalves as food (Foe and Knight 1986, Navarro et al. 1996). For Sphaeriidae, there are numerous studies dealing with the anatomy of alimentary tract, but only few reports or rather hypotheses about their feeding habits (Mackie and Flippance 1983, Raikow and Hamilton 2001). As sphaeriids occur in diverse habitats, the question was if their nourishment simply involves all ingestible and digestible particles that are present in their habitats or if particular species have some special preferences. Therefore, I tried to obtain some preliminary data by screening the stomach and intestine contents. The results, which might serve as a springboard to further detailed examinations, are reported in the Chapter 4. The main objectives of this part were to indicate:

What microorganisms do the sphaeriid clams ingest – is there any selection or is the process just mechanical?

What proportion of the ingested matter is really utilised as food and what proportion passes through the alimentary tract without being processed?

Chapter 1 – Kořínková, T., Morávková, A. 2010: Does polyploidy occur in central European species of the family Sphaeriidae (Mollusca: Bivalvia)? Centr. Eur. J. Biol. 5(6):777-784.

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VERSITA

Central European Journal of **Biology**

Does polyploidy occur in central European species of the family Sphaeriidae (Mollusca: Bivalvia)?

Research Article

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Abstract: Some representatives of the bivalve family Sphaeriidae are assumed to be polyploid. In this study, 11 sphaeriid species (nine of the genus *Pisidium*, one of *Musculium*, and one of *Sphaerium*) inhabiting central Europe were studied karyologically, 10 of them for the first time. Analysis revealed high chromosome numbers (from 140 to 240). To elucidate the origin of high chromosome numbers, DNA contents were measured by flow cytometry in 5 of the studied species and, for comparison, in *S. corneum* and *S. nucleus*, which are known to be diploid (2n=30). Species with high chromosome counts yielded very similar DNA contents that are not higher than in the related species with low diploid numbers. This finding contradicts a possible origin of these species by recent polyploidization or hybridization of related species. Chromosome complements of the investigated species with high chromosome numbers differ from those with low 2n in their small chromosome size and the high proportion of subtelo- or acrocentric chromosomes. This indicates their possible origin either by an ancient polyplodization event followed by chromosomal rearrangements or by multiple chromosome fissions.

Keywords: *C-value • Chromosome number • Chromosomal rearrangements • Flow cytometry • Palaeopolyploidy • Pisidium • Sphaerium*

1. Introduction

In molluscs, polyploidy has been reported in 5 gastropod families [1] and in the bivalve order Veneroida [2-4]. All these groups are hermaphroditic and in some of them either self-fertilization [1] or parthenogenesis [2] has been demonstrated. Within the Veneroida, triploidy occurs in some lineages of the genera *Lasaea* (Lasaeidae) [2] and *Corbicula* (Corbiculidae) [3,4] and high ploidy levels are typical for the freshwater family Sphaeriidae. The last group is characterized by a small body size (adults of some species do not exceed 2 mm) and viviparity (development of the larval stages in specialized nutritive tissues within the parent's gills). It comprises 3 genera: *Sphaerium* and *Musculium*, considered as closely related, and *Pisidium.* Up to now, only 15 sphaeriid species have been studied cytogenetically. Only 4 of them were considered diploid: *Sphaerium corneum* (2n=30 and 2n=36, respectively) [5-7, Kořínková, unpublished], *S. nucleus* (2n=30) (Kořínková, unpublished.), *S. rhomboideum* (2n=44) [8] and *S. nitidum* (2n=30) [7], The remaining 11 species exhibited chromosome counts from 100 to 247 [7]). Lee and Ó Foighil [9] considered the pattern of chromosome numbers within the *Sphaerium-Musculium* clade to be a consequence of ancient polyploidization, whereas within the genus *Pisidium* they found molecular evidence for recent autopolyploidization, based on the sequences of the multiple alleles of the single-copy gene for 6-phosphogluconate dehydrogenase. Most of the karyotype studies on sphaeriids have been carried out on polyploid taxa from America. *Pisidium casertanum* is the only Palaearctic species that had been studied previously [10,11].

Despite the occurrence of polyploidy in the family Sphaeriidae, there are no data on genome sizes in

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this group. In other bivalves genome size has been determined by various methods in over 100 species of 8 families. The C-values range from 0.69 pg in *Crassostrea virginica* (Ostreoida: Ostreidae) to 5.4 pg in *Acila castrensis* (Nuculoidea: Nuculidae). A veneroid clam closely related to Sphaeriidae, *Corbicula japonica,* exhibits a C value equivalent to 1.94 pg (measured by Feulgen densitometry) (Gregory, T.R., 2010: Animal Genome Size Database, http://www.genomesize.com).

The goal of the present study was primarily to collect karyological data from central European species in order to screen for the range and distribution of chromosome numbers. DNA contents were measured in selected species representing different points of the range, with the aim to test for a possible correlation between chromosome number and C-value.

2. Experimental Procedures

2.1 Material

Specimens of 11 species (9 of the genus *Pisidium*, 1 of *Musculium*, and 1 of *Sphaerium*) were collected from spring 2007 to spring 2009 at 16 sites listed in Table 1. When possible, at least 15 individuals were sampled from each site (using a bowl-shaped sieve), transported into the laboratory in small containers containing wet filter paper, and processed within three days of collection.

2.2 Preparation of chromosomes

Animals were incubated in 0.02% colchicine in tap water for 6 to 18 hours. In larger species (*M. lacustre* and *S. rivicola*), gonads were removed and used for chromosome preparations. In *Pisidium*, the preparations were made from the whole body except the foot and digestive tract. The tissues were hypotonised in 0.02% colchicine in deionised water for 30–35 min and fixed in two changes (5 and 15 min) of methanol: acetic acid (3:1- v:v) fixative. Finally, each piece was dissociated in a drop of 60% acetic acid on a microscope slide with the aid of fine tungsten needles. The slide was then placed on a hot plate heated up to 45°C and the suspension was smeared using a fine tungsten needle. Preparations were air-dried overnight, stained with 5% Giemsa solution in Sörensen phosphate buffer (pH 6.8) for 25 min, and inspected under an Olympus BX 50 microscope. Black-and-white images of chromosome plates were made with a CCD camera DP 71 (Olympus) and analysed using the program Cell D (Olympus). Well-spread prometaphases and metaphases of mitosis were used to count chromosomes. For each population, the highest recorded chromosome number was considered to represent the correct diploid number. When possible, rough sorting of the chromosomes into the morphological types was attempted. The classification was done according to the arm ratio (long arm/short arm) [12].

2.3 Flow cytometric analysis of nuclear DNA content

Nuclear DNA content was measured in 7 species with high chromosome counts (Table 2) and 2 species with a diploid genome exhibiting 2n=30 (*S. corneum* and *S. nucleus*). When possible, specimens were collected from the same population as used for chromosome preparations; populations from distal, hardly accessible or temporary habitats were replaced by more suitable ones. From each population, at least 2 specimens were measured on different days. Tissues were processed following the simplified two-step procedure version A for unfixed cells [13], with slight modifications. In brief, a small piece of tissue (typically about 20 mg), usually from the foot of individual, was first homogenised in Otto's Buffer I containing 0.1 M citric acid and 0.5% Tween [14], mixed, and filtered. After addition of 1 ml of Otto's buffer II (containing 50 µg of RNAse, and 50 µg of propidium iodide), the final mixture was incubated for 10–15 min in the dark at room temperature (RT) before analysis. The nuclear DNA content was measured by the flow cytometer LSRII (BD Biosciences) with acquisition software FACSDiva 6.1.2. Human leukocytes (*ca.* 10^6/ml), prepared in the same way as the sample, were used as a reference standard. Both external (separate measurements of the sample and standard) and internal standardization (sample and standard mixed together before staining) were tried out. The former method proved to be more suitable.

The measurements of the samples and standards followed at short intervals with the same FACS settings. Measurement of each sample was repeated at least twice. Multiple sets of samples were measured, each set on a different day. The C-values of species under investigation were then calculated using the peak means of the measured sample and the standard as follows:

DNA content (sample) = peak mean (sample)/peak mean (standard) x DNA content (standard)

For DNA content of the standard, the C-value of human male (3.22 pg, [15]) , was used.

3. Results

Chromosome numbers of the studied species are listed in Table 1. (Where a range is given, some of the plates could not be counted accurately.) The lowest chromosome number in a presumably complete mitotic metaphase was

Species	Collection site	Coordinates	Collection date	spec	mit	2n
M. lacustre	Emilie pond , Poodří PLA, Studénka, CZ	49° 42' 3" N, 18° 5' 55" E	2.5.2009 TK	5	75(29) [16;3;2;2;51]	210–214
M. lacustre	Weinlache, oxbow lake Neisse river, Görlitz, D	51° 8' 15" N, 14° 59' 34" E	20.6.2007 TK	3	17(3) [2;2;13]	210
M. lacustre	Neuteich Pond, Niederspree PLA, D	51° 24' 2" N, 14° 55' 90" E	30.7.2007, TK+HR	1	14(8)	210
P. amnicum	forest stream, Stobnica , PL	52° 43' 0" N, 16° 37' 0" E	23.4.2009 TK	2	3 [1;2]	>200
P. amnicum	Alba mill race, Týniště nad Orlicí, CZ	50° 9' 2" N, 16° 5' 22" E	26.5.2007 TK	1	2	214–230
P. casertanum	Ponědražský rybník pond, shallow edge of a fishpond, Ponědraž, CZ	49° 7' 11" N, 14° 42' 36" E	14.4.2007 TK	1	2	150
P. casertanum	drain "Peisker Graben", Niederspree PLA, D	51° 24' 10" N, 14° 54' 50" E	21.6.2007, TK+ HR	4	30 [2;2;3;23]	190–200
P. casertanum	Bohdanečský rybník pond, shallow edge of a fishpond, Bohdaneč,CZ	50° 5' 17" N, 15° 40' 24" E	19.5.2007 TK	2	16(9) [12;4]	180
P. globulare	Bažantnice, swamp in Poodří PLA, Studénka, CZ	49° 43' 16" N, 18° 6' 27" E	2.5.2009 TK	3	39	150–180
P. henslowanum	mill race, Ludwigsdorf, D	51° 13' 0" N, 15° 0' 12" E	31.7.2007 TK	1	2	190 (n = 95)
P. nitidum	Jizera river, Semily, CZ	50° 35' 16" N, 15° 20' 12" E	24.5.2007 TK	2	9	120–140
P. obtusale	Jezioro Dominickie lake, shallow edge of a lake, Boszkowo, PL	51° 57' 12" N 16° 20' 6" E	23.4.2009 TK	4	21(5) [9;1;3;7]	210–220
P. obtusale	Rod pond, shallow margin of a fishpond, Frahelž, CZ	49° 7' 11" N, 14° 44' 50" E	8.4.2007 TK	1	11	200
P. obtusale	Bohdanečský rybník pond, shallow edge of a fishpond, Bohdaneč,CZ	50° 5' 17" N, 15° 40' 24" E	19.5.2007 TK	1	1	204
P. personatum	Tiché údolí, swamp, Roztoky u Prahy, CZ	50° 9' 2" N, 14° 23' 13" E	27.10.2007 TK	3	18(10) [3;2;13]	210
P. milium	small drain near Kotvice pond, Poodří PLA, Studénka, CZ	49° 42' 22" N, 18° 4' 40" E	2.5.2009 TK	3	8 [3;2;3]	220
P. supinum	Vltava river, Praha, CZ	50° 5' 22" N, 14° 24' 41" E	13.5.2008, 25.6.2009, TK	3	43(24) [4;18;21]	160–170
S. rivicola	Vltava river, Praha, CZ	50° 5' 22" N, 14° 24' 41" E	25.6.2009, TK	3	11(8)	240 (n = 110–120)

Table 1. List of karyotyped species.

Data include: species, data on collection sites (name and brief description of the locality, nearest settlement, country, GPS coordinates, collection date, name of collector), data on the specimens investigated (spec. – number of specimens having dividing cells, mit. – number of analysable mitotic metaphases, in parentheses number of metaphases with complete chromosome number, in brackets number of mitoses obtained from the respective individuals, 2n – the supposedly complete chromosome number)
PLA = Protected Landscape Area, CZ = Czech Republic, D = Germany, PL = Poland,
TK =T. Kořínková, HR = H. Reise (Senckenberg Museum of Natural History, Görlitz).

found in *P. nitidum* (120–140, Figure 1-Part B – image b), the highest (240) in *Sphaerium rivicola* (Figure 1-Part C – image c).

Chromosome morphology could be distinguished only at a few late mitotic metaphases. All morphological types were present in all the species investigated; metacentric and submetacentric chromosomes usually made up about one third of the chromosome complement. In all species there were up to 3 larger meta- to subtelocentric chromosome pairs. It was not possible to group the remaining chromosomes into pairs of homologous chromosomes or groups of homeologous chromosomes

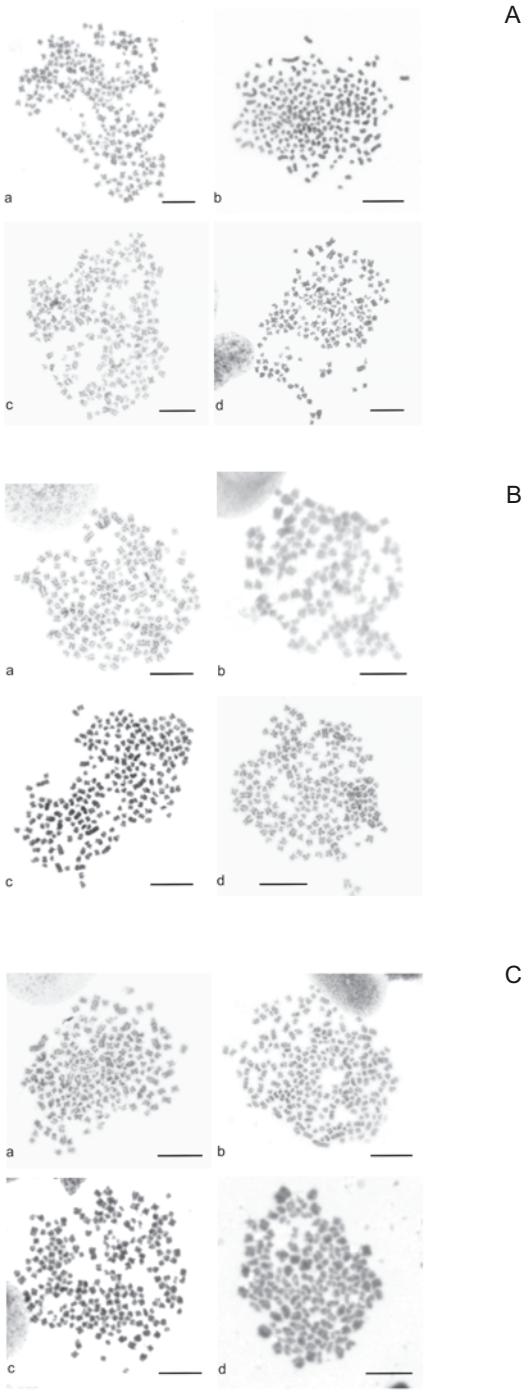

Figure 1. Central European species of the genera *Pisidium, Musculium* and *Sphaerium* – mitotic metaphases. Chromosome counts of the appropriate plates are presented in brackets.
Part A: (a) *P. amnicum* (240), Alba mill race, CZ; (b) *P. milium* (>210), Kotvice pond, CZ; (c) *P. casertanum* (195), Peisker Graben, D; (d) *P. globulare* (176), Bažantnice, CZ.
Part B: (a) *P. henslowanum* (190), Ludwigsdorf, D; (b) *P. nitidum* (140), Jizera river, CZ – the chromosome morphology is not distinguishable; (c) *P. obtusale* (220), Jezioro Dominickie pond, PL; (d) *P. personatum* (210), Tiché údolí, CZ.
Part C: (a) *P. supinum* (165), Vltava river, CZ; (b) *M. lacustre* (230), Neuteich pond, CZ; (c) *S. rivicola* (>230), Vltava river, CZ; (d) *S. rivicola* (diakinesis, *ca*. 120 bivalents), Vltava river, CZ.

Species	Collection site	2n	Number of specimens	CV(%)	1C (pg)
P. casertanum	Bohdanečský rybník pond, shallow edge of a fishpond, Bohdaneč, CZ	180	5	0.04	2.36+-0.16
P. personatum	Tiché údolí , swamp, Roztoky u Prahy, CZ	210	5	2.7	2.22+-0.32
P. amnicum	Bohdanečský rybník pond, shallow edge of a fishpond, Bohdaneč, CZ	230	3	0	2+-0.1
P. obtusale	Bohdanečský rybník pond, shallow edge of a fishpond, Bohdaneč, CZ	204	3	2.0	2.72+-0.06
P. supinum	river Vltava, Praha, CZ	160-170	3	0	2.55+-0.5
M. lacustre	Bohdanečský rybník pond, shallow edge of a fishpond, Bohdaneč, CZ	210	5	7.9	2.65+-0.21
S. corneum	river Vltava, Praha, CZ	30	6	4.4	2.61+-0.12
S. nucleus	Březina NR, small pond, Kostomlaty p./Mil., CZ	36	3	1.9	2.55+-0.05
S. rivicola	river Vltava, Praha, CZ	240	7	7.2	2.85+-0.69

Table 2. DNA contents of species studied.

Data include: brief reference to the collection site (see Table 1 for details), chromosome number, number of individuals measured, coefficient of variance, C-value.

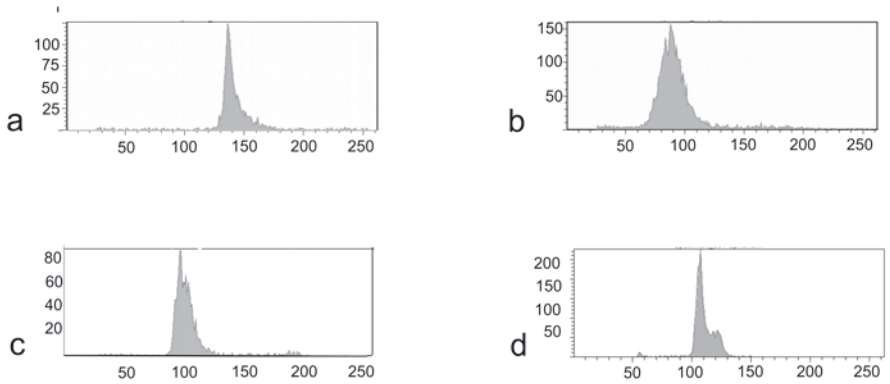

Figure 2. Relative DNA contents of studied species, flow cytometry histograms (x-axis: relative DNA content, y-axis: frequency of events): a – *S. rivicola*; b – *P. casertanum*; c – *P. amnicum*; d – *S. corneum*.

owing to their small and gradually decreasing size; the smallest chromosomes were less than 1 µm long.

In preparations of *S. rivicola*, over 30 plates of the first meiotic division (pachytene to metaphase I) were obtained, usually each with 110–120 bivalents. From late diplotene till metaphase I, chiasmata could be observed on the larger bivalents, usually only one, exceptionally two, per bivalent (Figure 1-Part C – image d).

From *P. henslowanum* (2n=190), 2 postpachytene plates with 95 bivalents and one incomplete diakinesis with chiasmate bivalents were obtained.

C-values are summarised in Table 2; selected measurements are compared in Figure 2. C-values of the species investigated (based on comparisons with human leukocytes as the reference standard) ranged from 2 (*P. amnicum*) to 2.85 (*S. rivicola*) pg

per cell. There was no significant correlation between chromosome number and C-value (Figure 3).

4. Discussion

The present study provides data on chromosome numbers and morphology of 11 bivalve species of the family Sphaeriidae and on C-values of selected species. All the 11 species investigated exhibit high chromosome numbers (140 in *P. nitidum* up to 240 in *S. rivicola*), which fall within the range of previously reported chromosome counts in sphaeriids (see [8] for an overview).

The chromosome number of *S. rivicola* (2n=240) observed in this study is the highest recorded within the genus *Sphaerium*. The striking difference in chromosome numbers between the *S. corneum* group (*S. corneum* and *S. nucleus* 2n=30 [6]) and representatives with high 2n (more than 200 chromosomes) raises the question of monophyly of the genus. Molecular data corroborate its subdivision into 4 groups [16] and place *S. rivicola* into the proximity of North American species of the subgenus *Amesoda*, rather than in a close relationship with the *S. corneum* group. However, even *Sphaerium* species with strikingly different chromosome numbers can be closely related, provided that polyploidization was involved in speciation within the genus. Petkeviciute *et al*. [8] hypothesize on the basis of karyotype and molecular data that *S. rhomboideum* (2n=44) represents a diploid ancestor of polyploid *S. occidentale* (2n=209–213). The *Sphaerium* species investigated in this study, despite having different chromosome numbers, showed very similar DNA contents. Their C-values were close to that of a diploid representative of the related family Corbiculidae, *Corbicula japonica* (C=1.94 pg, Gregory, T.R., 2010: Animal Genome Size Database, http://www.genomesize.com), in which 2n=38 [17]. The C-values thus do not imply any recent polyploidization taking place among the investigated species. However, identification of ancient polyploids on the basis of genome size comparison is complicated because of the phenomenon of genome downsizing, which occurs in many groups of organisms after a polyploidization event [18,19] and might be a possible explanation for the situation found in Sphaeriidae throughout the course of this study. The genome size can undergo significant reductions even in newly arisen polyploids [20]. In paleopolyploids, the situation is often further complicated by the absence of the diploid progenitor [19]. Indeed, our data suggest that if extant diploid ancestors of *S. rivicola* exist at all, they must be found outside the *S. corneum* group.

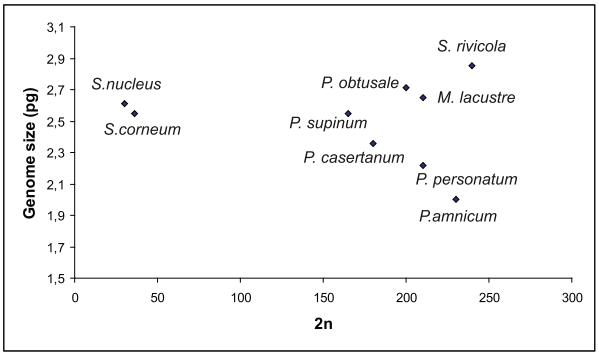

Figure 3. Diagram illustrating the C-values of the 7 selected species plotted against their chromosome numbers. Note the absence of any relationship between the variables.

Considering the genus *Pisidium*, the estimates of chromosome numbers in *P. casertanum* from 3 populations in the Czech Republic (180, 190 and 200, respectively) agree with the data reported in [10] and [11] (180 and 190 chromosomes, respectively). The difference in chromosome counts between our and earlier studies (*ca*. 10 chromosomes) can be regarded as intraspecific variability or as an artefact resulting from counting incomplete or poorly spreaded mitotic plates. It would be highly interesting to compare the C-value of the *S. casertanum* population exhibiting 2n=150 with those of the populations possessing 180 or 190 chromosomes. Unfortunately, this population was no longer available for the flow cytometric analysis. Among the 9 *Pisidium* species investigated, neither chromosome numbers nor DNA contents gave any evidence for some of the karyotypes being derived from another one by a single, recent polyploidization event, such as that reported by Lee and Ó Foighil [9] in *P. dubium* and *P. adamsi*. As in *Sphaerium*, also among the Palaearctic representatives of the genus *Pisidium* no potential ancestor species with low chromosome number has so far been found.

Another approach to determine the level of polyploidy of an organism is to analyse the meiotic pairing of chromosomes. Polyploidy can cause formation of multivalents, univalents and other pairing irregularities in meiosis. However, meiotic cells can exhibit some correction mechanisms that facilitate regular formation of bivalents, especially in allopolyploids, by enforcing homologous pairing and hindering homoeologous pairing [21,22]. Thus, correct pairing of chromosomes in meiosis can be achieved through evolution and the course of meiosis in paleopolyploids is usually the same as in diploid organisms (diploidization). Interestingly, metaphase I plates of *S. rivicola* seemed to consist mainly or exclusively of bivalents, whose number roughly corresponded to half the chromosome number in somatic cells (110–120 bivalents = *ca*. 240 mitotic

chromosomes). However, owing to the condensation and small size of these bodies, it cannot be excluded that some of them are univalents. An analogous situation was found in *P. henslowanum*, although the observation was based only on a limited number of meiotic plates.

An alternative to the hypothesis on polyploidy is suggested by the fact that sphaeriid species with high chromosome numbers differ from *S. corneum*, *S. nucleus* and *S. rhomboideum* in their smaller chromosome size and higher proportion of uniarmed (subtelocentric or nearly acrocentric) chromosomes. The length of chromosomes in mitotic metaphase ranges from 3 μm to almost 13 μm in *S. corneum* [6] and 2 to 6 μm in *S. rhomboideum* [8], whereas in the species with high chromosome numbers it was usually about 1 μm. The predominance of small uniarmed chromosomes in karyotypes of species with high 2n points out to multiple chromosome fissions. These could have acted in the karyotype evolution and caused an increase of chromosome number by splitting one biarmed chromosome into two uniarmed. Alternatively, if the previously discussed old polyploidization event was involved in the ancient history of the sphaeriid clade, it might have been followed by chromosomal rearrangements, evolutionary diploidization and reduction of the genome.

Many examples of clades that have diverged from a common polyploid ancestor by genome rearrangements are known in various organisms, especially in angiosperms (see [23,24] for references). Lee and O´Foighil [9] proposed this mechanism as the most probable scenario for phylogeny of the North American *Sphaerium-Musculium* clade. The present study suggests that this phenomenon may also be typical for the Palaearctic sphaeriid species. However, a more detailed phylogenetic analysis would be necessary to evaluate precisely the relationships among the species investigated and to elucidate the role of polyploidization and chromosomal rearrangements in the evolution of their karyotypes.

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into the PhD. thesis of Mgr. Tereza Kořínková. The proportion of co-authors work is mentioned in paragraph „Acknowledgment" of this work – Alena Morávková did all FACS measurements and partial analysis, while Tereza Kořínková did all other work, preparing of the experiments, collection, cytogenetic preparations and their evaluation, processing of samples and she prepared the manuscript of the paper.

Prague 8th November2010

RNDr. Alena Morávková, Ph.D.

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Structure and meiotic behaviour of B chromosomes in *Sphaerium corneum/S. nucleus* complex (Bivalvia: Sphaeriidae)

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Abstract Karyotypes of eight populations of *Sphaerium corneum* and two populations of *S. nucleus* (Bivalvia: Sphaeriidae) from central Europe were compared. The basic set of these hermaphroditic molluscs is formed by 30 biarmed autosomes and exhibits only slight interpopulational variation in morphology. These differences are not species-specific. One pair of nucleolar organiser regions was detected by silver staining. The prophase and metaphase of the first meiotic division is highly modified in both species. Pachytene is followed by a diffuse stage, characterized by decondensation of chromosomes and by enhanced metabolic activity. The diffuse stage has not been reported in bivalves so far. Bivalents of the following stages are achiasmatic both in the testicular and ovarian part of the gonad. The two species are further peculiar for occurrence of B chromosomes, which is a rare phenomenon in organisms with achiasmatic meiotic systems. The small metacentric B chromosomes exhibit intra- and interindividual variability in number, they show irregular meiotic pairing and segregation (formation of bivalents or univalents), and possess larger proportional amount of constitutive heterochromatin than the A chromosomes. Interestingly, the B chromosomes also undergo decondensation during the diffuse stage like A chromosomes which may indicate their transcriptional activity.

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Keywords Achiasmatic meiosis · B chromosome · Bivalve · Constitutive heterochromatin · Diffuse stage · Sphaerium

Introduction

The term "B chromosomes" denotes all supernumerary chromosomes, that are not essential for the organism, vary in number within cells of the individual, between individuals and between populations. They neither pair nor recombine with chromosomes of the standard complement of the species (A chromosomes), show non-Mendelian inheritance owing to the irregular segregation during mitotic and meiotic divisions, are usually heterochromatinized, and bear in general no active genes. They arise by different processes—namely from the A chromosomes (especially sex chromosomes) by nondisjunctions and following structural rearrangements, or they have interspecific origin from hybridization events (see Jones 1995; Camacho et al. 2000 for a review).

The B chromosomes can be regarded as typical selfish genetic elements pursuing the goal of their own dispersion (Jones 1991), as they are known to change the pairing and recombination pattern of the A chromosome set (Parker et al. 1990; Jones et al. 1991), act as sex ratio distorters (Beladjal et al. 2002; Beukeboom and Werren 1992) and show accumulation during mitosis and meiosis (Parker et al. 1989; Jones 1991). Their effects on the fitness of the host organisms range from negative as e.g. in human (Fuster et al. 2004) to positive (Jones 1995 and references therein). In most cases the B chromosomes have no observable effect on the external phenotype (Camacho et al. 2000). Being maintained for many generations under a considerable pressure of selection due to their frequent

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negative influence on fitness, the B chromosomes undergo many structural changes which make it difficult to trace their origin (Green 1990).

Among molluscs, only several occurrences of B chromosomes have been reported so far. They have been found in some gastropod genera (Evans 1960; Patterson 1969, 1973) and in the bivalve order Veneroida (Insua and Thiriot-Quiévreux 1992; Leitão et al. 2008; Ó Foighil and Thiriot-Quiévreux 1991, 1999). Another unusual characteristic of the order Veneroida is the occurrence of polyploidy in some genera (Lee 1999; Ó Foighil and Thiriot-Quiévreux 1999; Park et al. 2000; Lee and Ó Foighil 2002). In the veneroid family Sphaeriidae, high chromosome numbers are considered a consequence of ancient allopolyploidization events (Lee and Ó Foighil 2002; Kořínková and Morávková 2010). Up to now, only two sphaeriid species are considered diploid—the North American *Sphaerium rhomboideum* (Petkevičiūtė et al. 2007) and the Palaearctic *S. corneum*. The latter probably comprises a complex of cryptic—thus morphologically scarcely distinguishable—species (Petkevičiūtė et al. 2006). As an effect, there are some contradictory data on its chromosome number. The first report on a karyotype of a bivalve, determined as *S. corneum*, comes from Keyl (1956), who has found a complement formed by 36 chromosomes. He has also reported achiasmatic meiosis in the testicular part of the gonad of this species. Further studies have revealed a variability of chromosome numbers in populations of *S. corneum* complex. Baršienė and Baršytė Lovejoy (2000) have reported $2n = 30$ in *S. corneum* and *S. nitidum* and the occurrence of aneuploidy, which they considered as an effect of environmental pollution. Petkevičiūtė et al. (2006) have found karyotype dimorphism in a population of "*S. corneum*" (which more likely belonged to the sister species *S. nucleus*—Petkevičiūtė pers. com.). In one of the sympatric karyotypes, they have also found small metacentrics, which exhibited intra- and interindividual variability of number, and assigned them as B chromosomes. The present study aims to compare the karyotypes, meiosis, pattern of constitutive heterochromatin, and the distribution of nucleolar organiser regions (NOR) in central European populations of *S. corneum* and its sibling species, *S. nucleus*, with special respect to the characteristics of B chromosomes.

Materials and methods

Specimens from 10 populations (eight of *S. corneum*, two of *S. nucleus*) were collected from summer 2006 to spring 2009 at sites listed in Table 1. When possible, at least 15 individuals were sampled from each site (using a bowl-shaped sieve), transported to the laboratory in small containers containing wet filter paper and karyotyped within 3 days of collection.

Living animals were placed into a 0.02% solution of colchicine in tap water for 6–18 h. Subsequently, pieces of the hermaphroditic glands were dissected out in deionised water (without appropriate distinction of the testicular and ovarian part), hypotonised in 0.02% solution of colchicine in deionised water for 30–35 min and fixed in two changes (5 and 15 min) of methanol: acetic acid (3:1 v:v) fixative. Finally, each piece was dissociated on a microscope slide in 60% acetic acid using a pair of fine tungsten needles.

Table 1 Collection sites, including the name of the locality, the nearest settlement, country and coordinates of the locality

Collection site	Code	Coordinates	Date of coll., name
Sphaerium corneum			
Bechyňský potok—stream, Záluží, CZ	BE	49°14′N; 14°39′E	IV. 2007 TK + LJ
Pools in the Labe river floodplain, Čelákovice—Sedlčánky, CZ	CE	50°10′N; 14°48′E	30.5. 2007 LJ
Weinlache—Neisse river cutoff, Görlitz, D	GO	51°8′N; 14°59′E	20.6.2007 TK
Maškův mlýn—mill race, Praha, CZ	MA	49°59′N; 14°19′E	16. 11. 2005, 28.5.2006, 13. 7. 2006 TK
Stream Rokytka, Praha, CZ	RO	50°04′N; 14°36′E	4.6.2008 TK
Sánský kanál—artificial drain, Křečkov, CZ	SA	50°11′N; 15°05′E	12. 8. 2008 TK + RP + GS
Weißer Schöps stream, Kunnersdorf, D	SC	51°13′N; 14°55′E	30.7.2007 TK + HR
Vltava river, Praha, CZ	VL	50°05′N; 14°25′E	14.5. 2007, 30.7. 2008 TK
Sphaerium nucleus			
Small pond at Březina national reserve, Kostomlaty pod Milešovkou, CZ	BR	50°33′N; 13°54E	1. 7. 2009 LJ
Drain near Kotvice pond, Studénka, CZ	PO	49°42′N; 18°05′E	8.7. 2007, 16. 11. 2008, 2.5. 2009 TK + LJ

Country codes: CZ—Czech Republic, D—Germany. Collectors: HR—H. Reise (State Museum of Natural History, Görlitz, Germany), LJ—L. Juřičková (Charles University, Praha, Czech Republic), RP + GS—R. Petkevičiūtė + G. Stanevičiūtė (Institute of Ecology, Vilnius, Lithuania), TK—T. Kořínková

The slide was placed on a histological hot-plate heated to 40–45°C and the suspension was smeared using a fine tungsten needle. Preparations were air-dried overnight, stained with 5% Giemsa solution in modified Sörensen phosphate buffer (66 mM KH_2PO_4 and 26 mM Na_2HPO_4, pH = 6.8) for 25 min, and inspected under an Olympus BX 50 microscope. Black-and-white images of chromosome plates were made under objective 50× or 100× (immersion lens) with a CCD camera DP 71 (Olympus). For construction of the karyotype, five optimum spread late mitotic metaphases of one specimen were chosen from each population. At this stage, mitotic chromosomes have clearly separated chromatids joined only in the centromere region and thus enabling the distinction of centromere. Chromosome morphology was classified according to the arm ratio (long arm/short arm) (Levan et al. 1964). Relative chromosome length was calculated as a percentage of the total chromosome length of the diploid set.

To avoid destruction of chromatin, preparations for C-banding and silver staining were prepared at lower temperature (35–38°C) of the histological hot-plate. The C-banding of unstained slides was performed according to the procedure of Král et al. (2008), with some modifications. The preparations were dried for 2–3 h between each step and stained with 5% Giemsa in Sörensen phosphate buffer (pH 6.8) for 45 min. The preparations were then stained with 5% Giemsa solution in modified Sörensen phosphate buffer (pH = 6.8) for 35 min.

Silver staining was performed on selected unstained slides or slides stained by Giemsa including some C-banded preparations. The method of Howell and Black (1980) was used, with some modifications. Seven drops of 50% $AgNO_3$ solution and three drops of 1% gelatine solution (containing 1% formic acid) were placed onto the preparations, mixed together using a glass rod and covered with a cover slide. Preparations were transferred to a hot-plate heated to 50°C. After 5 min of incubation on four layers of cellulose cotton wool, the preparation was placed into a current of tap water to remove the cover slide and to rinse the preparation. The preparations were air-dried overnight at room temperature.

Results

Mitotic karyotypes

Thirty-three (27 of *S. corneum*, six of *S. nucleus*) out of more than 100 animals collected at 10 sites provided over 300 mitotic metaphase plates usable for the evaluation of chromosome number and morphology.

All populations possessed a complement of 15 autosome pairs gradually decreasing in size (Fig. 1a) Their morphology was predominantly metacentric but with one to three submetacentric pairs (usually pairs Nos 4, 7 and 12, see Table 2). Metaphase plates with less than 30 autosomes, which were found sporadically on the preparations from almost all specimens, were regarded as random artefacts originating during hypotonization and spreading of chromosome plates rather than aneuploidies and were excluded from further considerations.

Silver staining revealed the presence of an intercalary nucleolar organiser region on the chromosome pair no. 14 (Fig. 1a). Karyotypes of all populations except *S. corneum* from the Vltava river also included a varying number (1–6) of small (relative length of each less than 1% of the diploid set) metacentric B chromosomes (Table 3). In mitosis, these chromosomes did not exhibit any differences from A chromosomes with respect to condensation, chromatid separation or intensity of staining. They could only be distinguished from chromosomes of the A set on the basis of markedly smaller size. As the colchicine blocks the most cells at metaphase, it was not possible to observe segregation of B-chromosomes in mitotic anaphase.

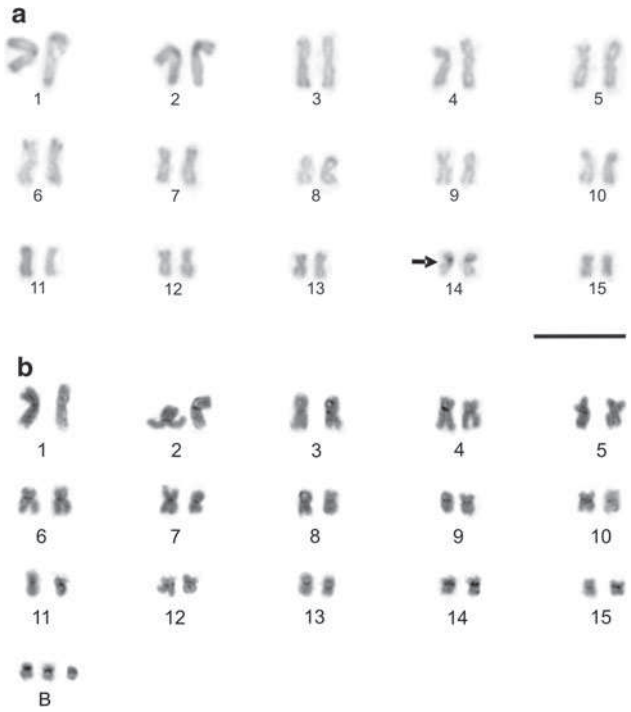

Fig. 1 Karyotypes of *Sphaerium corneum*; based on mitotic plates. **a** *S. corneum*, VL population, mitotic karyotype (silver staining), $2n = 30$, no B chromosomes. The pair no. 14 bears an intercalary NOR (*arrow*). **b** *S. corneum*, SA population, mitotic karyotype (C-banding), $2n = 30 + 3B$. Note the blocks of heterochromatin at centromere regions of all chromosomes. The three B chromosomes are smaller than that of any A chromosome pair and their blocks of heterochromatin are comparatively larger. See Table 1 for abbreviations of localities. Bar = 10 μm

Table 2 Characteristics of autosome pairs of central European populations of *S. corneum* and *S. nucleus*

Pair no.	Population									
	BE	CE	GO	MA	RO	SA	SC	VL	BR	PO
1	1.18 ± 0.1	1.22 ± 0.03	1.2 ± 0.05	1.24 ± 0.16	1.16 ± 0.04	1.12 ± 0.1	1.19 ± 0.03	1.1 ± 0.05	1.26 ± 0.14	1.26 ± 0.11
	12.54 ± 0.28	6.25 ± 0.33	11.74 ± 0.88	11.38 ± 0.72	12.17 ± 0.59	12.2 ± 0.8	11.16 ± 0.34	11.74 ± 0.36	12.61 ± 0.6	10.7 ± 0.2
	m	m	m	m	m	m	m	m	m	m
2	1.24 ± 0.16	1.34 ± 0.05	1.29 ± 0.12	1.27 ± 0.11	1.31 ± 0.18	1.2 ± 0.17	1.25 ± 0.11	1.14 ± 0.04	1.06 ± 0.09	1.24 ± 0.14
	10.44 ± 0.22	5.32 ± 0.22	10.42 ± 0.83	9.7 ± 0.36	10.6 ± 0.1	10.64 ± 0.24	10.32 ± 0.46	10.46 ± 0.36	10.58 ± 0.45	9.66 ± 0.21
	m	m	m	m	m	m	m	m	m	m
3	1.33 ± 0.25	1.27 ± 0.11	1.24 ± 0.22	1.56 ± 0.12	1.22 ± 0.14	1.2 ± 0.09	1.14 ± 0.09	1.33 ± 0.24	1.21 ± 0.15	1.32 ± 0.1
	8.34 ± 0.18	4.51 ± 0.17	9.34 ± 0.51	8.6 ± 0.46	9.35 ± 0.4	9 ± 0.5	8.76 ± 0.12	9 ± 0.46	8.21 ± 0.42	8.26 ± 0.28
	m	m	m	m	m	m	m	m	m	m
4	1.75 ± 0.26	1.33 ± 0.14	1.14 ± 0.14	1.7 ± 0.24	1.59 ± 0.38	2.14 ± 0.7	2.2 ± 0.58	1.38 ± 0.16	1.69 ± 0.31	1.81 ± 0.16
	8.54 ± 0.16	4.16 ± 0.08	8.34 ± 0.21	8.04 ± 0.34	8.25 ± 0.06	8.08 ± 0.34	7.86 ± 0.7	8.24 ± 0.42	8.35 ± 0.54	7.76 ± 0.21
	sm	m	m	sm	m	sm	sm	m	sm	sm
5	1.25 ± 0.05	1.31 ± 0.17	1.28 ± 0.14	1.54 ± 0.1	1.41 ± 0.15	1.66 ± 0.34	1.26 ± 0.07	1.42 ± 0.12	1.77 ± 0.25	1.25 ± 0.09
	7.74 ± 0.1	3.93 ± 0.08	7.51 ± 0.27	7.4 ± 0.24	7.52 ± 0.3	7.8 ± 0.48	7.92 ± 0.14	7.66 ± 0.3	7.12 ± 0.67	7.6 ± 0.1
	m	m	m	m	m	m-sm	m	m	m	m
6	1.36 ± 0.21	1.49 ± 0.22	1.64 ± 0.33	1.78 ± 0.13	1.48 ± 0.06	1.27 ± 0.16	1.37 ± 0.23	1.5 ± 0.13	1.19 ± 0.19	1.28 ± 0.19
	7.22 ± 0.17	3.39 ± 0.08	7.17 ± 0.33	6.72 ± 0.22	6.81 ± 0.43	7.16 ± 0.42	7.42 ± 0.22	6.86 ± 0.14	7.33 ± 0.25	7.2 ± 0.12
	m	m	m-sm	sm	m	m	m	m	m	m
7	1.37 ± 0.4	1.22 ± 0.21	1.22 ± 0.1	1.68 ± 0.19	1.53 ± 0.24	1.7 ± 0.35	1.28 ± 0.08	1.4 ± 0.05	1.3 ± 0.18	1.22 ± 0.03
	6.62 ± 0.17	3.12 ± 0.11	6.11 ± 0.12	6.18 ± 0.2	6.21 ± 0.13	6.22 ± 0.38	6.64 ± 0.1	6.38 ± 0.14	6.93 ± 0.18	6.92 ± 0.13
	m	m	m	sm	m	sm	m	m	m	m
8	1.31 ± 0.28	1.43 ± 0.38	1.51 ± 0.17	1.46 ± 0.12	1.4 ± 0.17	1.67 ± 0.28	1.17 ± 0.33	1.43 ± 0.43	1.22 ± 0.23	1.3 ± 0.21
	6.16 ± 0.09	2.8 ± 0.09	5.8 ± 0.19	5.68 ± 0.36	5.8 ± 0.24	5.98 ± 0.46	6.04 ± 0.22	6.26 ± 0.28	6.79 ± 0.13	6.6 ± 0.09
	m	m	m	m	m	m	m	m	m	m
9	1.89 ± 0.16	2.17 ± 0.44	1.33 ± 0.18	1.4 ± 0.46	1.4 ± 0.23	1.52 ± 0.16	1.76 ± 0.35	1.24 ± 0.22	1.21 ± 0.12	1.46 ± 0.25
	5.52 ± 0.11	2.64 ± 0.04	5.51 ± 0.22	5.34 ± 0.18	5.51 ± 0.13	5.48 ± 0.46	5.86 ± 0.4	5.8 ± 0.24	6.5 ± 0.19	6.28 ± 0.13
	sm	sm	m	m	m	m	sm	m	m	m
10	1.41 ± 0.11	1.28 ± 0.34	1.15 ± 0.07	1.19 ± 0.14	1.11 ± 0.08	1.31 ± 0.14	1.18 ± 0.18	1.25 ± 0.1	1.17 ± 0.21	1.66 ± 0.37
	5.58 ± 0.08	2.57 ± 0.04	5.38 ± 0.18	5.08 ± 0.18	5.35 ± 0.14	5.22 ± 0.44	5.52 ± 0.12	5.56 ± 0.18	5.55 ± 0.88	5.96 ± 0.06
	m	m	m	m	m	m	m	m	m	m
11	1.41 ± 0.13	1.18 ± 0.15	1.35 ± 0.14	1.28 ± 0.07	1.26 ± 0.33	1.59 ± 0.31	1.14 ± 0.07	1.64 ± 0.35	1.61 ± 0.44	1.34 ± 0.17
	5.04 ± 0.07	2.34 ± 0.06	5 ± 0.3	4.76 ± 0.14	5.2 ± 0.23	4.9 ± 0.32	5.1 ± 0.14	4.96 ± 0.14	5.74 ± 0.31	5.62 ± 0.25
	m	m	m	m	m	m	m	m	m	m

Table 2 continued

Pair no.	Population									
	BE	CE	GO	MA	RO	SA	SC	VL	BR	PO
12	1.38 ± 0.23	1.33 ± 0.11	1.22 ± 0.09	1.25 ± 0.13	1.27 ± 0.17	1.51 ± 0.25	1.43 ± 0.26	1.53 ± 0.13	2.62 ± 0.31	2.33 ± 0.3
	4.6 ± 0.08	2.11 ± 0.1	4.72 ± 0.29	4.38 ± 0.2	4.89 ± 0.08	4.56 ± 0.5	4.72 ± 0.08	4.78 ± 0.1	4.37 ± 0.29	4.38 ± 0.22
	m	m	m	m	m	m	m	m	sm	sm
13	1.88 ± 0.36	1.41 ± 0.26	1.07 ± 0.13	1.33 ± 0.09	1.27 ± 0.18	1.56 ± 0.08	1.28 ± 0.07	1.31 ± 0.23	1.1 ± 0.1	1.2 ± 0.09
	4.28 ± 0.26	1.97 ± 0.1	4.33 ± 0.14	4.04 ± 0.1	4.43 ± 0.18	4.02 ± 0.18	4.3 ± 0.24	4.38 ± 0.14	3.25 ± 0.42	3.82 ± 0.18
	sm	m	m	m	m	m	m	m	m	m
14	1.29 ± 0.05	1.34 ± 0.27	1.12 ± 0.12	1.32 ± 0.27	1.16 ± 0.16	1.19 ± 0.08	1.47 ± 0.08	1.31 ± 0.25	1.49 ± 0.23	1.43 ± 0.16
	3.94 ± 0.08	1.82 ± 0.06	3.87 ± 0.25	3.64 ± 0.1	4.11 ± 0.16	3.52 ± 0.28	3.96 ± 0.16	3.92 ± 0.24	2.5 ± 0.27	3.28 ± 0.12
	m	m	m	m	m	m	m	m	m	m
15	1.27 ± 0.08	1.31 ± 0.06	1.13 ± 0.13	1.16 ± 0.1	1.26 ± 0.2	1.22 ± 0.08	1.22 ± 0.2	1.4 ± 0.21	1.17 ± 0.17	1.4 ± 0.09
	3.4 ± 0.09	1.5 ± 0.14	2.19 ± 0.8	2.3 ± 0.4	3.85 ± 0.21	3.16 ± 0.5	3.46 ± 0.12	3.68 ± 0.34	2.1 ± 0.22	2.8 ± 0.05
	m	m	m	m	m	m	m	m	m	m

First line within the cell—arm ratio (average ± SD) calculated as length ratio long arm/short arm. Second line within the cell—relative length of the pair in % of total length of the diploid set (average ± SD). Third line within the cell-morphology of the particular A chromosome pair. See Table 1 for abbreviations of localities. *m* metacentric, *sm* submetacentric chromosomes

Course of meiosis

Thirteen animals from seven populations (five of *S. corneum*, two of *S. nucleus*) provided numerous plates of the first meiotic division. All specimens exhibited achiasmatic meiosis only, diplotene and diakinesis being replaced by postpachytene stage with parallel alignment of homologues and with absence of chiasmata. In total, 134 postpachytene and metaphase I plates were suitable for detailed analysis.

The length of pachytene bivalents reached up to 30 µm. Only few pachytene nuclei were spread out enough to allow distinction of the particular bivalents. In some pachytene nuclei, association of bivalents by their centromeric regions was observed (Fig. 2b). Centromeres were often visible as positively heteropycnotic dots on the late zygotene and pachytene bivalents (Fig. 2a, b) and at the beginning of the following stage. At this stage, the homologous chromosomes seemed to be partially desynapsed (Fig. 2c.). It was not possible to study the details of this process due to the insufficient spreading of nuclei. This transitional stage was followed by an extreme decondensation of all chromosomes. At this peculiar stage, the bivalents became almost undistinguishable (Fig. 2d). These plates were less numerous than those of pachytene and the stages that followed after the period of chromatin decondensation, which implies a relatively short duration of chromatin decondensation. After recondensation of the chromatin (Fig. 2e), the bivalents entered postpachytene stage being still achiasmatic (Fig. 2f, g). The 30 chromosomes of the A complement formed 15 regular bivalents. The chromosomes composing bivalents lay in parallel and their primary constrictions could be distinguished (Fig. 2g), as the centromeric regions were never completely aligned. The homologous chromosomes formed numerous twists along the longitudinal axis of the bivalent (Fig. 2e–h). This feature was most apparent on the largest bivalents, morphology of the smaller ones could not be properly observed. During the following contraction at prometaphase and metaphase I the chromosomes showed a gradual straightening. They became closely aligned except for the centromeric region (Fig. 2h, i). The supernumeraries were present at postpachytene and metaphase I cells both as univalents and as bivalents. These bivalents were with all probability achiasmatic, though their morphology could not be properly distinguished due to their small size (Table 3). The univalents were present both in the plates with even and odd number of B chromosomes. Neither pairing nor association between the A and B chromosomes was found. During late metaphase I, the segregation of the bivalents, which attained their maximum condensation and minimum length, began with a formation of a longitudinal gap between the homologues (Fig. 2j). It proceeded from the centromeric region towards the distal parts. As a result, the segregated

Table 3 *S. corneum* and *S. nucleus*: distribution of numbers of B chromosomes and characteristics of their pairing at postpachytene and metaphase I

Species	Specimen code	n_B—number of B chromosomes ($2n = 30 + n_B$)							Number of meiotic bivalents									
		0	1	2	3	4	5	6	15	16	17	18	15 + I	15 + I + I	15 + I + I + I	15 + I + I + I + I	16 + I	17 + I + I
*Sphaerium corneu*m	BE	6	1	2		1								1					
	CE1								7	2	3			1	2				
	CE2	2	1					1	1	1			1	4				2	
	CE3			4	2	1	1		2		5								
	GO1	5		1	1	2	4	4											
	GO2			1	2	4	1	4											
	MA1	8	2	2															
	MA2	4	1	3					9	2	1	7							
	MA3		5																
	MA4	1	4	3					5	1			10						
	MA5	15	2	2					5				5						
	MA6		2																
	RO1	1							1										
	RO2		1							5				1					
	RO3	43	4																
	SA1	1		3		3													
	SA2	4		3	1			1								1			
	SA3	4																	
	SA4	1	2																
	SA5																		
	SC1	8	2	1															
	SC2	4		1	1														
	SC3								3	1	2								
	VL1	4																	
	VL2	8		1															
	VL3	5																	
	VL4	3																	
Sphaerium nucleus	BR1	1			2	1		1	1	1	2	4	2	10		10		2	1
	BR2	1	2	4															
	BR3	2	7	3		1													
	PO1													3		3			
	PO2		2	1		2													
	PO3	1									3								

Left part of the table: number of mitotic plates showing the particular numbers of B chromosomes (the number of A chromosomes was 30 in all cases). Right part of the table: number of meiotic plates showing number of bivalents and univalents in the particular specimens. I = univalent (e.g. 15 + I stands for 15 bivalents and 1 univalent). See Table 1 for abbreviations of localities

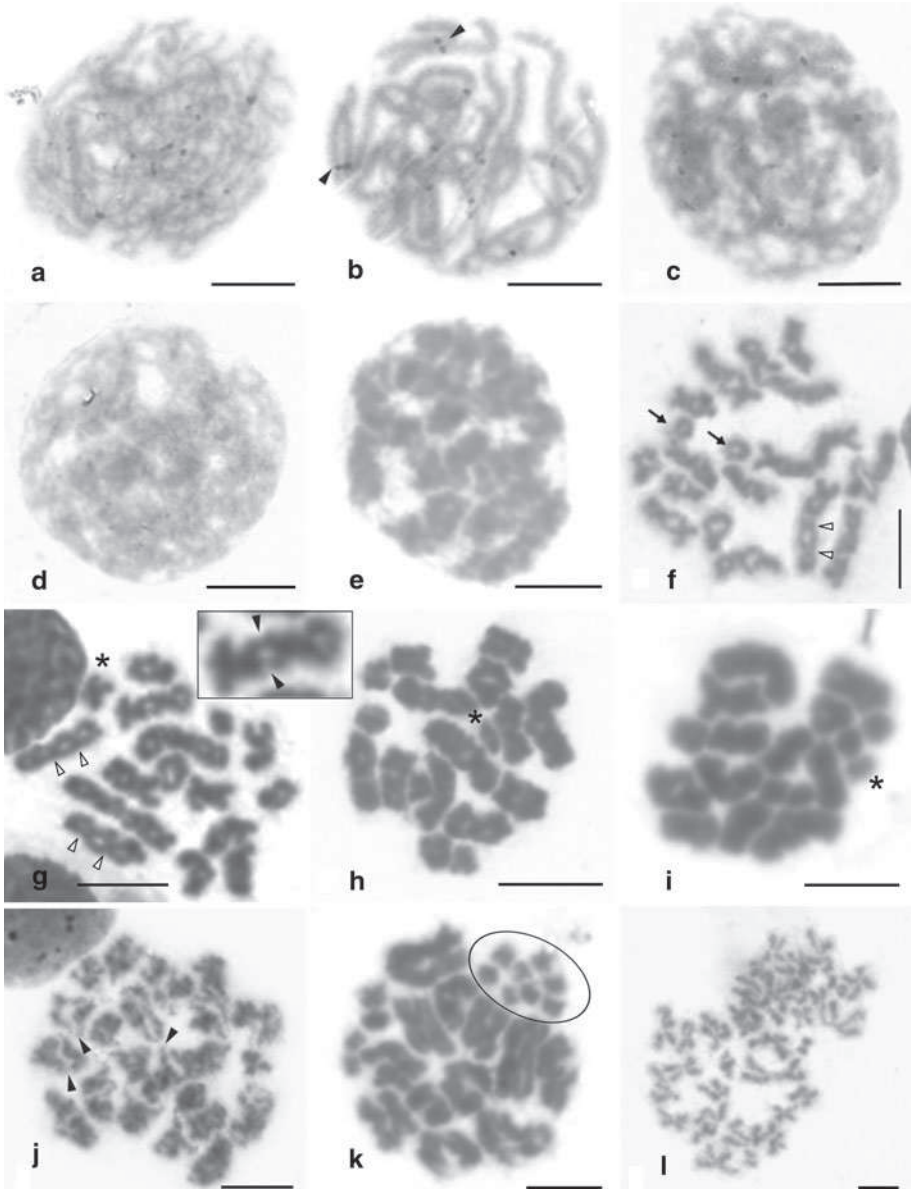

Fig. 2 *Sphaerium corneum* and *S. nucleus*, course of meiotic division. **a** *S. corneum*, CE population, zygotene. **b** *S. corneum*, CE population, late pachytene, centromeres are observable as *dark spots*; *arrowhead*—association of two bivalents by centromeric regions. **c** *S. corneum*, CE population, transition pachytene/diffuse stage, the bivalents are not clearly delimited but their centromeres are still positively heteropycnotic. **d** *S. corneum*, CE population, diffuse stage. **e** *S. corneum*, BE population, transition diffuse stage/postpachytene. Note the fuzzy outlines of the recondensing bivalents. **f** *S. corneum*, MA population, postpachytene consisting of 17 bivalents (*arrow*—bivalents formed by B chromosomes). Note the numerous twists (one of them delimited by *open arrowheads*) on the longer chromosomes. **g** *S. corneum*, MA population, early postpachytene containing 15 bivalents and one univalent (*asterisk*). *Open arrowheads* as in (**f**). *Inset* Enlarged bivalent with arrowheads showing the primary constrictions. **h** *S. nucleus*, PO population, early metaphase I containing 17 bivalents and 1 univalent (*asterisk*). The bivalents are shorter and more straight than in the previous phase. **i** *S. nucleus*, BR population, early metaphase I, 16 bivalents and 1 univalent (*asterisk*). **j** *S. corneum*, CE population, latest metaphase I with 16 bivalents. *Arrowheads*—homologue separation proceeding from the centromeric regions. **k** *S. nucleus*, BR population, transition metaphase/anaphase I. The homologues lie in parallel. *Encircled*—precociously separated B chromosome univalents forming a group on the periphery of the plate. **l** *S. corneum*, MA population, metaphase II, two sister plates. See Table 1 for abbreviations of localities. Bar = 10 μm

homologues were oriented more or less in parallel at metaphase/anaphase I transition (Fig. 2k). B chromosome bivalents, where present, exhibited precocious segregation. B chromosomes formed a group on the periphery of some plates at metaphase I and metaphase/anaphase I transition (Fig. 2k).

Concerning the following meiotic stages, only few metaphases II were found (Fig. 2l), which were not spread enough to yield any information on segregation of the B chromosomes during the second meiotic division.

C-banding pattern

Centromere regions of all A chromosome pairs were formed by relatively small (5–10% of the total chromosome length) blocks of constitutive heterochromatin (Fig. 1b). In some mitotic metaphases telomeric blocks were observed at both ends of the pair no. 4 (not shown). The pericentromeric bands of the B chromosomes were approximately as large as those on A chromosomes, however, they made up to 25% of the B chromosome length due to the small size of these chromosomes.

Discussion

Basic karyotype characteristics of the *Sphaerium corneum* complex

All 10 examined populations of *S. corneum* and *S. nucleus* possess very similar A chromosome sets, each with 15 metacentric to submetacentric pairs of gradually decreasing length. This similarity can reflect their close relatedness as well as the small karyotype variability in bivalves, where species within one genus or even family often have the same chromosome number and number of chromosome arms (Patterson 1969; Nakamura 1985; Thiriot-Quiévreux 2002). However, previous studies also indicated some karyotype variability in the *S. corneum* group (Keyl 1956; Petkevičiūtė et al. 2006). They have reported from Germany (Keyl 1956) and Lithuania (Petkevičiūtė et al. 2006) populations with a diploid number of 36 chromosomes including some acrocentric chromosome pairs. Such a karyotype has not been revealed in any of populations in the present study.

We found B chromosomes in almost all populations of *S. corneum* and *S. nucleus*. In mitosis, they could only be distinguished from the A chromosomes on the basis of their small size. Their number showed both intra- and interindividual variation within the populations, from one to six. For populations from Lithuania, Petkevičiūtė et al. (2006) have reported even up to 10 B chromosomes.

One of important karyotype characteristics is the distribution of constitutive heterochromatin. The C-banding technique has so far been applied only to 16 bivalve species (Leitão and Chaves 2008), mainly of the economically important families Mytilidae, Ostreidae and Pectenidae. When detected, the constitutive heterochromatin was mostly pericentromeric. Our study presents first information on a C-banding pattern in Sphaeriidae. Like the previously studied bivalves, *S. corneum* and *S. nucleus* showed pericentromeric C-bands on all chromosomes, but small telomeric blocks were also detected in some populations. The pericentromeric bands were small in all chromosomes of the A set and relatively larger in the B chromosomes.

Silver-staining revealed a subterminal NOR at short arms of the submetacentric chromosome pair no. 14. This result is in compliance with the observation of a secondary constriction on this chromosome pair (Petkevičiūtė et al. 2006). Presence of one pair of NOR is considered as ancestral in bivalves (Skuza et al. 2009 and references therein).

The course of meiosis

The course of prophase and metaphase of the first meiotic division was complicated in both species.

Following pachytene the gametocytes entered a peculiar stage characterized by a larger size of nucleus and by an extreme chromatin decondensation. It probably represents the so-called "diffuse stage", which has not been previously reported in bivalves, but it has been described in various other animals (Benavente and Wettstein 1980; Šťáhlavský et al. 2006) and plants (Klášterská 1977). This period exhibits a considerable decondensation of chromatin and an enhanced metabolic activity. It occurs more frequently in female meiosis, probably in connection with an intense synthesis of reserve substances in the developing oocytes (Benavente and Wettstein 1980). However, it has also been reported from male meiosis (e.g. Šťáhlavský et al. 2006). In the preparations from *Sphaerium* which is a simultaneous hermaphrodite, it was not possible to distinguish between the male and female meiotic plates and it is thus not clear in which of them the diffuse stage occurs. Diffuse stage has also been found in another species of the family, *S. rivicola*, where it is followed by a stage corresponding to a standard diakinesis with chiasmate bivalents (Kořínková—unpublished).

Unlike in *S. rivicola*, in the populations of *S. corneum* and *S. nucleus*, the further course of meiosis after the emergence from the diffuse stage is with all probability achiasmatic. Chiasmata are not formed and the homologous chromosomes lie in parallel (postpachytene stage). Achiasmatic meiosis has already been reported by Keyl (1956) from the testicular part of *S. corneum* gonad. The numerous twists of homologous chromosomes observed by Keyl (1956) and recently by ourselves at postpachytene are quite an unusual phenomenon. Similar structures have been reported from the achiasmatic male meiosis of the tsetse fly *Glossina morsitans* (Craig-Cameron et al. 1973; Davies and Southern 1977) where it probably contributes to the

homologue association in the presence of an incomplete synaptonemal complex. It cannot be concluded on the basis of light microscopy, whether such a function can also be assigned to the coils in *Sphaerium corneum* and *S. nucleus*. However, a similar coiling of bivalents after emergence from the diffuse stage has also been observed by Král et al. (2006) in males of the haplogyne spider genus *Loxosceles*, where the bivalents are chiasmatic. This suggests the twisting and coiling of homologous chromosomes might alternatively be just a consequence of the rapid recondensation of large bivalents after an extreme decondensation during the diffuse stage.

In a related bivalve species *Sphaerium rivicola*, which exhibits a high chromosome number (2n ca. 240, Kořínková and Morávková in 2010), chiasmata are still present. However, their manifestation is delayed up to the stage which most probably corresponded to a standard diakinesis (Kořínková, unpubl.). It is thus possible that meiosis in particular lineages of Sphaeriidae attains different degrees of evolution towards an achiasmatic mechanism. Such transitional modes of meiosis (cryptochiasmatic meiosis) have already been reported in some insects (White 1973).

In bisexual species where achiasmatic meiosis has been found, it is confined to the heterogametic sex (White 1973; John 1990). Achiasmatic meiosis is less common in hermaphroditic organisms. Similarly to gonochorists, it occurs usually only in one (either male or female) part of the gonad (e.g. Oakley 1982). However, it can occur both in spermatogenesis and oogenesis as found in oligochaetes of the family Enchytraeidae (Christensen 1961). We found achiasmatic bivalents in all late prophase I and metaphase I plates. Given the spermatogenesis and oogenesis in *Sphaerium* usually occur simultaneously (Okada 1935; Kořínková, unpublished data) and considering that we used both the ovarian and testicular part of the gonadal tissue without their separation, it can be inferred in *S. corneum* and *S. nucleus* the meiosis is probably achiasmatic both in the male and female part of the gonad.

Structure and meiotic behaviour of B chromosomes

C-banding revealed that the B chromosomes in *S. corneum* and *S. nucleus* have a larger proportion of constitutive heterochromatin than the chromosomes of the A set. From various organisms, there are many examples of B chromosomes consisting completely (Feldberg et al. 2004; Coluccia et al. 2004) or for the most part (Fagundes et al. 2004; Vujošević and Blagojević 2004) of constitutive heterochromatin. On the contrary, the B chromosomes of some organisms do not show predominance of constitutive heterochromatin (Bertolotto et al. 2004; Kartavtseva and Roslik 2004), which is also the case of *Sphaerium*.

B chromosomes can be subjected to facultative heterochromatinization, which is manifested by positive heteropycnosis during whole cell cycle or at some of its stages (e.g. John and Hewitt 1965). Such a phenomenon was not found in our populations of *Sphaerium*.

The B chromosomes exhibited both intra- and interindividual variability in number. We suppose the observed intraindividual variability to be a consequence of irregularities in mitotic segregation. Unfortunately, neither the previous studies nor the present one yielded observations on mitotic segregation of the B chromosomes in *Sphaerium*. This might have resulted from the use of colchicine which blocks cells at metaphase. The interindividual variability in B chromosome number results from their irregular segregation at gonial mitoses or at meiosis (e.g. the observed clustering of segregated B chromosomes at anaphase I).

The behaviour of B chromosomes at meiosis is an attractive subject to study. In chiasmatic meiosis, an increase of B chromosome number leads to increased chiasma frequency of A chromosomes (Parker et al. 1990; Camacho et al. 2004; Leach et al. 2004). As the increase of chiasma frequency is rather detrimental to the organism, it might be one of the aspects urging the organisms to eliminate the B chromosomes. In achiasmatic systems like our *Sphaerium* species the B chromosomes cannot influence chiasma frequency, which might facilitate the organism to tolerate B chromosomes. Nevertheless, reports on the occurrence of B chromosomes in organisms with achiasmatic meiosis are very scarce. Small telocentric B chromosomes have been reported in the monoecious plant genus *Fritillaria* (Liliaceae) (Noda 1975), where male meiosis is achiasmatic. The behaviour of B chromosomes was similar in the achiasmatic male and chiasmatic female part: they formed achiasmatic bivalents or multivalents and they resolved into univalents earlier than autosomal bivalents did. At achiasmatic male meiosis in the tsetse fly *Glossina austeni*, B chromosomes behave as univalents irrespective of their number (Craig-Cameron et al. 1973). In chiasmatic females of the same species, B chromosomes form bivalents when two or more are present (Davies and Southern 1977). Interestingly, microchromosomes (m-chromosomes) in the hemipteran genus *Saldula* also behave differently in chiasmatic and achiasmatic forms. Where male meiosis is chiasmatic, synapsis of the m-chromosomes is normal but they then undergo desynapsis. Conversely, in the achiasmatic forms synapsis of the m-chromosomes is maintained (Nokkala and Nokkala 1983).

The B-chromosomes of *Sphaerium* are either present as univalents or pair (but never with A chromosomes) forming achiasmatic bivalents. No twists or coils have been observed on the B bivalents during postpachytene. This might, however, be a mere consequence of the small size of the B chromosome bivalents. The presence of B

chromosome univalents is apparently not an artefact caused by the method of preparation (e.g. colchicine treatment or/and exposition of fixed cells to higher temperatures). In that case the pairing would be affected both in A and B chromosomes, but in our preparations all A chromosomes formed only bivalents. The regular formation of bivalents by the A chromosomes as well as a lack of associations between the A and B chromosomes indicate that B chromosomes of *Sphaerium* do not influence substantially the meiotic pairing of A chromosomes. The B chromosome bivalents exhibit a precocious separation at anaphase I, similarly as already observed by Noda (1975) in *Fritillaria*.

Possible origin and role of B chromosomes in *Sphaerium*

Remarkably, B chromosomes found in our populations of *S. corneum* and *S. nucleus* do not comply with all characteristics of B chromosomes. On one hand they show intraindividual and interindividual variation in number due to their mitotic instability and the irregularities in meiotic pairing and segregation. This can be documented by the presence of univalents at postpachytene and metaphase I as well as by the precocious separation of the B chromosome bivalents and by clustering of the segregated B chromosomes at anaphase I. However, the B chromosomes in *Sphaerium* were neither formed completely or from a large part by constitutive heterochromatin nor were inactivated by facultative heterochromatinization. Indeed, they exhibited the same condensation and pycnotic cycle as standard chromosomes during mitosis and meiosis, including diffuse stage. The despiralisation of B chromosomes at diffuse stage indicates that they can be transcribed like A chromosomes. The absence of heterochromatinization of B chromosomes and their despiralisation at diffuse stage might be alternatively explained by a relatively recent origin of these chromosomes, so that they are not yet subjected to inactivation.

The origin of B chromosomes in the *S. corneum* complex is not yet resolved. Petkevičiūtė et al. (2006) hypothesized about origin of B chromosomes in *Sphaerium* by Robertsonian translocations between A chromosomes. They have found at the same locality a population with 36 A chromosomes (putative ancestral karyotype) and an other population with 30 A chromosomes plus varying number of B chromosomes. The former karyotype included two subtelo-acrocentric pairs whereas in the latter all chromosomes were meta-submetacentric (like in the populations studied by us), which would comply with the possible evolution by Robertsonian translocations.

Another mechanism which could also play some role in the origin of B chromosomes in *S. corneum*/*S. nucleus* complex is interspecific hybridization. Hybridization events were probably involved in the karyotype evolution of other species and genera of the family Sphaeriidae, which posses much higher chromosome numbers (2*n* more than 100) and are probably polyploid (Lee and Ó Foighil 2002). A more detailed study including a more extensive sampling, measurement of fitness-related characteristics, and the use of molecular techniques (analysis of chromatin modifications and search for the active genes on B chromosomes and their function) would be needed to investigate the possible function of B chromosomes in *Sphaerium*.

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A COMPLEX VIEW OF BREEDING STRATEGY AND LIFE-HISTORY IN ONE POPULATION OF *SPHAERIUM CORNEUM* LINNAEUS 1758 (BIVALVIA: SPHAERIIDAE)

Sphaerium corneum breeding strategy

TEREZA KOŘÍNKOVÁ

Abstract

A population of viviparous freshwater bivalve *Sphaerium corneum* from an artificial mill race subjected to irregular changes of water level was investigated using quantitative monthly sampling, paraffin histological sections and chromosome preparations. Unlike most of the previously studied populations of the genera *Sphaerium* and *Musculium*, the one presented in this paper exhibits less synchronised life-spans. Despite two main birth periods, many specimens release their broods also individually during the season. An adult usually breeds at least twice in a season. The life span is more than one, more often one and half year. All age and length classes are able to overwinter. Spermatogenesis and oogenesis apparently proceeds simultaneously from spring to autumn and it occurs also in fully developed larvae which are still retained inside their parents´ gills. Relationship between the observed breeding strategy and the fluctuations of environment was discussed, as well as the intraspecific and interspecific variation in life-history traits within Sphaeriidae.

Key-words: *Sphaerium*, life-history, gametogenesis, precocious maturation

INTRODUCTION

Hermaphroditic, primarily freshwater clams of the family Sphaeriidae are unique among bivalves – they possess true viviparity. This is why reproductive biology of the genera *Sphaerium, Musculium* and *Pisidium* has been attracting attention since more than 100 years - see Table 1 for summarized data on the first two genera and Heard (1965; 1977) for information on *Pisidium*. The most studies on sphaeriid reproduction and ecology have been carried out from 1960s to early 1980s.

The viviparity in these organisms is achieved by development of larval stages in the cavities of their parent's inner demibranches, where special nutritive and protective structures are present. Fully developed young individuals, the so-called extra-marsupial larvae (Okada, 1935b), which are already self-sustaining (Beekey *et al*., 2000), are retained until the conditions are favourable for their release. Two general breeding strategies can be distinguished with respect to the individual life-span: semelparity (only one brood per year or per entire life cycle - see Heard, 1977) and iteroparity (more than 1 brood in the individual life span), the former is typical for some species of the genus *Pisidium* s. l. while the latter for *Sphaerium* s.l. (Heard, 1965; Heard, 1977). The reproductive mode of the population depends both on individual breeding strategies and environmental conditions, as demonstrated e.g. by

Mackie *et al*. (1976), Mackie (1979) or Mackie & Flippance (1983). Although the reproduction of both the semelparous and iteroparous individuals can occur continuously from spring to late autumn, the gonad activity of the individuals and accordingly the hatching of young clams is to a large extent synchronised and confined usually to two short periods of the season, in spring and autumn. As shown by field and laboratory experiments (Thomas, 1963; Gale, 1977), newborn individuals of some species (particularly genus *Musculium*) can grow very fast and complete the whole life span within 1 or 2 months but, on the other hand, they can also cease to grow for few months during periods of hibernation or aestivation (Thomas, 1963; Mackie *et al*., 1976; Mackie, 1979; Hornbach *et al*., 1982). Individuals born in spring usually reach maturity, reproduce in autumn and either die after first reproduction, leaving overwintering generation of the offspring, or they hibernate as adults and reproduce again in the next year. Individuals born in autumn usually overwinter and reproduce in the next year.

The individual life span can range from several months, usually with one period of reproduction (born in spring and reproducing in autumn or born in autumn and reproducing in spring) to more than 1 year (undergoing at least 2, but usually more reproductions in 2 subsequent seasons). An interesting shift of this general pattern of reproduction was noted by Foster (1932) in a population of *S. striatinum*, where the main period of maximum reproduction has been found from November to January and the second peak took place in August.

The reproduction and breeding strategies of the most common European fingernail clam, *S. corneum*, has been studied already by Thiel (1924; 1926) and revised by Heard (1977). However, none of the authors has applied a complex approach combining regular sampling and evaluation of population structure over longer period of time with histological and cytogenetical investigations. The aim of the present study thus was a detailed investigation of one population of *S. corneum* chosen as a model, using combination of the above mentioned methods.

MATERIAL AND METHODS

Clams were sampled from an old mill-race connected to Radotínský brook, near Maškův mlýn ca. 2 km SW of Praha-Radotín, Český kras Protected Landscape Area. The water was running, with short periods of stagnation in the summer months and with irregular fluctuations of the water level caused by opening/closing of the lock gate. Bottom contained silt, leaves and organic material. Width of the channel 1 m, max. depth 0. 5 m. The characteristics as measured with YSI 556 MPS multiprobe (YSI Inc., Yellow Springs, USA) in September 2004 and April 2005 were as follows: water temperature 7.1°C (autumn)/ 13.1°C(spring), conductivity 1012 /1043 μs/cm^2, pH 7.9 (autumn)/ 9(spring), saturation by oxygen ranged from 63% after summer stagnation to 138 % in spring.

From February 2004 to July 2005 (except for months when the site was covered with ice and snow), monthly samples containing usually 50 to 100 specimens each were obtained by dredging the bottom within a randomly chosen square 0.5x0.5 m using a bowl-shaped sieve. The specimens (further referred to as “adults” to be distinguished from larvae) were transported into the laboratory. They were killed by a short immersion into carbonated water, fixed in 70% ethanol, measured with a slide calliper to the nearest 0.1 mm and dissected under a stereomicroscope (magnification 16 to 25 times). The following characters were measured: adult shell length, number of brood pouches, number, shell length and ontogenetic stage of the larvae in each brood pouch. Larvae, which attained earlier developmental stage and reached less than 50% (arbitrary chosen limit) of shell length of their kin in the same

brood pouch were considered as retarded. For the further analyses, both adults and larvae were divided into length classes (within a range of 1 mm for adults and 0.2 mm in the case of larvae). The data were processed using MS Excel and Statistica 6.0 (Statsoft Inc.) to obtain the following descriptive statistics: average and median shell length of the adults, number and percentage of adults in the respective length classes, percentage of gravid (i.e. bearing larvae of any developmental stage) adults in each monthly sample, number of larvae within the respective length classes (evaluated per 100 adults).

From each sample, 5 adults of different shell length (range 5-10 mm) were examined karyologically: gonads were dissected out and hypotonised for 30 min. in deionised water, fixed in 3 changes (5 min each) of methanol:acetic acid (3:1 v:v), dissociated with the aid of fine tungsten needles on a microscope slide in a drop of 60% acetic acid and then spread on the slide, placed on a hot plate (40°C), using one fine tungsten needle.

Besides the quantitative samples, 2 to 5 additional specimens were sampled each month for histological sections. These animals were killed and fixed in 70% ethanol for a few days and the thicker shells were removed by hand. Thin and fragile shells were dissolved by immersion into Bouin´s fixative for 24-48 hours and the specimens were then rinsed in 70% ethanol to remove the rests of picric acid.

In both cases, the soft tissues were then dehydrated by successive immersion in 96% ethanol (6-12 hrs. according to the size of the animal), propanol (2x 6-12 hrs.), propanol-methyl benzoate (3-6 hrs.), methyl benzoate (6-12 hrs.), benzene (12-24 hrs.), benzene-paraffin (12-24 hrs.) and finally paraffin. After stiffening, the blocks of paraffin were sectioned at 1-10 (usually 5 μm) using Leica- microtome, stained (after removal of the paraffin using xylene and series of 96%-80%-60%-40% ethylalcohole) in Masson´s triple stain and, after dehydration in propane and xylene, fitted in Canada Balsam.

RESULTS

POPULATION SIZE AND COMPOSITION

In 2004, periods of population size (approximately estimated from the sample size) decrease (by 50%) were recorded – in March, May and December (Fig 1). In March and May, they were followed by immediate increase of the population size and changes in the distribution of length classes in favour of the smallest postlarval individuals.

Shell length of free-living animals ranged from 2.5 (smaller newborn clams) to 12 mm. For average and median shell lengths and distribution of the length classes in monthly samples see Figs 1 and 2, respectively. All length classes in the range 3-9 mm were present throughout the year. Shell length of the most newborn clams ranged from 3 to 4 mm, newborns of the length class 2-2.9 mm were less numerous. The length class 3-3.9 mm was the most frequent one from March to August 2004 and also in June and July of the following season. Its maximum frequency was observed in June, when it together with the 2-2.9 length class made up more than 60% of the population, less prominent peak in the occurrence of smallest clams occurred in March-April. Adults with shell length between 6 and 9 mm were present in all monthly samples; their smaller percentual proportion in June-August was an effect of rapid increase in population size after the release of newborns rather than of dying out of the parental generation. In autumn, the proportion of the 6 to 9 mm length classes again increased, as the generation born in summer grew larger.

The largest individuals (shell length > 9 mm) were the less numerous during the whole period, except May 2005, when the last 3 length classes together made up more than 30% of the population. However, both this maximum and those reached in June and November 2004

were followed by immediate decline in number of these large individuals. In October, percentage of the largest individuals started increasing again and remained unchanged (6-7% of the population) until next spring. Most empty shells found at the collection site had length under 5 mm of above 9 mm, which implies that these size groups exhibit the highest mortality.

GRAVIDITY AND FECUNDITY

Percentage of all gravid adults (bearing larvae of any developmental stage) in the monthly samples ranged from 24% (June 2004) to 95% (March 2005) (Fig 3). Brood pouches with developing F1 embryos were found already in clams of the length class 3-3.9 mm, where the average percentage of gravid individuals reached 13.5 % and the average number of F1 per adult was low – 0.07. Gravid individuals of this length class occurred scattered throughout the whole season. Individuals larger than 7 mm (including those short after death, the tissues of which have not decayed yet) were all gravid and average number of larvae per adult increased up to 17.7 in the length class 10-10.9. Maximum number of brood pouches found in one adult was 12, which contained altogether 45 F1 embryos and larvae.

Embryos and larvae of different developmental stages were usually found within demibranches of an adult. During autumn and winter months the total number and percentual proportion of the youngest developmental stages increased substantially (Fig 4).

Shell length of the extramarsupial larvae (EML) ranged from 2.4 mm to 3.6 mm. They were found in number from 1 to 7 per one parent, only from April to August in individuals larger than 7 mm. Maximum percentage of individuals with EML was reached in May (22% in 2004 and 29% in 2005), followed by a rapid decline (Fig 3). In July and August 2004, only few (12 and 6.7 per 100 adults, respectively) EML were still present in demibranches of the parental generation, in autumn they were completely absent, which implies either completion of their development and release from their parents´ organisms, or death of the large parental organisms together with their descendants.

Percentage of retarded larvae was under 5% in the most monthly samples (Fig 3).

GAMETOGENESIS

The course of meiosis is achiasmatic (Keyl, 1956; Kořínková and Král 2010) with a diffuse stage (Kořínková and Král 2010). Chromosome preparations containing meiotic divisions were obtained from animals collected from May to July. In May, mainly pachytene and diffuse stages were found, whereas postpachytene and metaphase I stages predominated in material from June and July. In July, sporadic occurrences of meiosis II plates (prophase and metaphase II) were also noticed. A few pachytene stages were obtained also from July samples and few postpachytene plates from November samples, which implies a possible second, smaller, peak of gametogenesis in autumn. As the ovarian and testicular part of the gonad were usually smashed together on preparation of chromosome plates, it was not possible to distinguish between spermatogenetic and oogenetic meiotic divisions.

Histological sections revealed in the most specimens collected throughout the season (February - December) simultaneous occurrence of oogenesis and spermatogenesis. The oogonia and primary oocytes in the ovarian part were present in all monthly samples, but most abundant from February to April and again from September to December. Analogically, spermatogonia, spermatocytes and spermatids appeared in all monthly samples, however, spermatozoa were most numerous in February and September-October. All the adults

undergoing gametogenesis were at the same time bearing larvae of various developmental stages. In one EML found inside a parent in April, ovarian part of the gonad was found, though no oocytes were probably present yet.

DISCUSSION

The study brought valuable data on breeding and life-histories of one thoroughly sampled population of *S. corneum*.

Adult animals release their first brood at the shell length of 7 mm and the young postlarval individuals are born at length of 2-4 mm. This corresponds to the previous findings from another population by Thiel (1924). The present results also corroborate this author´s estimate of the life span duration (8-15 months) and the observation of two main birth periods in one year.

In the examined population, the distribution of shell lengths throughout the season also implies two birth periods (April and June), more or less coincident with dying of older animals. Nevertheless, the parental generation is never fully replaced by the offspring. Firstly, part of the young adults with shell length 7-8mm probably continues growing after their first brood to produce at least one more litter either in the same season or after overwintering and secondly, reproduction of some small proportion of individuals is probably not synchronised with the rest of population. This results in overwintering of animals of all sizes and ages and in a broad overlap of generations. A similar pattern was revealed e.g. by Zumoff (1973) in a population of *S. simile*, on the basis of histological sections and dissections of small monthly samples.

On the contrary, in other previously examined sphaeriid species, mostly from temporary waters of North America, the parental generation often completely dies out within few days after giving birth to the offspring (Foster, 1932; Thomas, 1963; Mackie, 1979; Way *et al*., 1980). The collection site under investigation was an artificial mill-race. Unlike in natural, flooded or drying-out habitats, the fluctuations of water current, water level and other characteristics caused by economical exploitation might be to a large extent unpredictable. The heterogeneity of breeding strategies in the population might be a sort of “bet-hedging” as a response to the varying conditions.

Comparison between the two seasons of investigation suggests that the shift of the coldest period to February in the year 2005 could have partly delayed both the growth, production of the early-spring litter and death of the largest individuals. In 2004, January was the most severe month (see the website of the Czech Hydrometeorological Institute, http://www.chmi.cz/meteo/ok/infklim.html for maximum, minimum and mean temperatures), whereas in February the temperatures were usually above zero and the most clams were active. In 2005, relatively mild (though with snow and ice cover) January was followed by cold February, which made a monthly collection impossible. The data from March 2005 did not indicate such an important increase in number of the youngest clams, as that in March 2004.

The meiotic divisions, which culminate in May- July, followed by substantial increase in number of the earliest embryonic stages in September-October, imply gamete production and fertilization in autumn. Similarly as reported by Okada (1935a) for *S. heterodon*, also the gonads of *S. corneum* contain immature gametes throughout the whole season, though there are certain peaks in production of primary oocytes and spermatozoa. Primary oocytes probably undergo following divisions after fertilization, as suggested by Okada (1935c) and Woods (1931).

Large proportion of embryos in brood pouches in February and March 2004 could be explained either by continuation of fertilization till winter, or by interrupted development of the F1 during winter due to hibernation. It seems probable that the most individuals underwent multiple fertilisations during one autumn-winter period. This corresponds to the formation of more brood pouches of differential developmental stage and to the production of two (exceptionally even more?) litters in the following season. In some animals the gametogenesis can also have different timing, as evident from the occurrence of meiotic divisions in chromosome preparations from November. The small (3-4 mm) clams, which are already gravid short after hatching probably undergo gametogenesis during the last stages of larval development. This phenomenon of the so-called precocious maturation in *S. corneum* was already described by Thiel (1928) and Heard (1977), who found mature gametes in histological sections of EML. Though no meiotic chromosome plates were found in larvae from this locality, they were scarcely present in chromosome preparations from other populations (Kořínková, unpublished), and evidence for well-developed gonads in the EML was found in the course of this study. Fertilization of such precociously mature individuals supposedly occurs immediately after birth, if not already within the parent´s demibranches by sperm of the own sibs. Self-fertilization might also take place, as evident from the arrangement of the genital tract (Woods, 1931; Okada, 1935a) and from the simultaneous occurrence of mature ova and sperm (Woods, 1931; Heard, 1965; this study). Precocious maturation is thus another aspect contributing to the heterogeneity of breeding strategies in the population – individuals born in spring and becoming gravid immediately can produce their first litter already in summer of the same season.

Differences in growth and developmental stage might be encountered among individuals of the same age within one brood pouch. This so-called larval retardation has been already found by Foster (1932), who noticed that in brood pouches containing higher number of embryos, some are usually considerably smaller. Mackie & Flippance (1983) suggested that the suppressed larvae not only reach smaller size, but also earlier developmental stage, and most of them probably die or never mature. The authors supposed the suppressed larvae to be “sacrified” for their kin to grow larger.

Hetzel (1993) opined that growth suppression of some larvae in comparison with their kin in the brood pouch also represents one strategy to increase temporal heterogeneity of reproduction: on favourable conditions, retarded individuals may continue growth and development up to the extramarsupial stage. The population examined in this study exhibited quite low percentage of retarded larvae (at most 5%), the frequency of which increases as the growth and development of the brood pouch and most enclosed larvae proceeds. Considerable size variation (by ca. 50%) was not exceptional even among extramarsupial larvae. This would support the hypothesis of Hetzel (1993).

The present study gave a detailed view of breeding strategy of one population inhabiting a small drain with seasonal fluctuations of some conditions. The population differs from the most previously studies populations of 8 species of the genera *Sphaerium* and *Musculium* by considerable variation of individual breeding strategies. This is accomplished by a life span of up to 1 and half year (compare with other population of *S. corneum*, Thiel 1924), combined with overwintering at any stage, capability of precocious maturation and probably also self-fertilization and ability to produce more broods at various times of the season. Sphaeriids exhibit variation in life-history tactics on specific (Heard, 1965; Mackie *et al.*, 1976; Way et *al.*, 1980) and generic (Heard 1965; 1977) level. Thus, in the case of such an euryvalent species as *S. corneum*, examination of more different populations by a combination of quantitative sampling with histological sections and chromosome preparations will be desirable to describe variation of its life-history traits.

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TABLES AND FIGURES

Table 1

Species	postlarval life-span (months)	birth periods	hib.	est.	broods per life	Reference
Musculium lacustre	6 months to 1 year	summer, autumn	J		1	Mackie, 1979
Musculium partumeium	13 (late spring-late spring)	late spring	J	J		Way et al., 1980
Musculium partumeium	13 (spring/next summer)	VI/VII, IX/XI				Way et al., 1980
Musculium partumeium	12-13 (summer/summer)	summer before dry period	J	J		Thomas, 1963
Musculium securis	6 (spring-fall, fall-spring) to 15 months	early summer, autumn	J	J	1- 2	Mackie, 1979
Musculium securis	VII-early VIII of next year, more than 1 year	late VII	A	J		Mackie et al., 1976
Musculium securis	1 year	VII-VIII, IX				"
Musculium securis	1 year	continuos, mainly fall-winter and VI				"
Sphaerium corneum	4-8 (spring-fall, fall-spring)	IV, VIII/IX	J			Thiel, 1924
Sphaerium fabale	12(summer-summer, fall-fall), 15 (summer-next fall)	early summer, late fall	A, J		1	Mackie, 1979
Sphaerium rhomboideum	14 (spring-next summer, summer-next fall)	spring-fall, peak in VII	A,N,		2	Mackie & Flippance, 1983
Sphaerium rhomboideum	12 (fall-fall)	whole season, with 3 maxima	A, J		1-2	Mackie & Flippance, 1983
Sphaerium simile	18-24	whole season, mainly summer and winter	A,J			Avolizi, 1971 cit in Zumoff, 1973; Zumoff, 1973
*Sphaerium solidulum**	12	winter, VIII	J, A			Foster, 1932
Sphaerium striatinum	12 (spring-spring, autumn-autumn)	IV/VII, VIII/X	A, J			Hornbach et al., 1982

* redetermined as *S. striatinum* by Hornbach et al. 1982

Table 1 – Overview of studies on life-histories of *Sphaerium* and *Musculium* species: hib. – hibernating stages, est. –aestivating stages, A – adult, J – juvenile (=postlarval stage short after release from the parent´s body), N – newborn; months of the year indicated by Roman numerals, Arabic numerals indicate duration of life-span in months

Figure 1

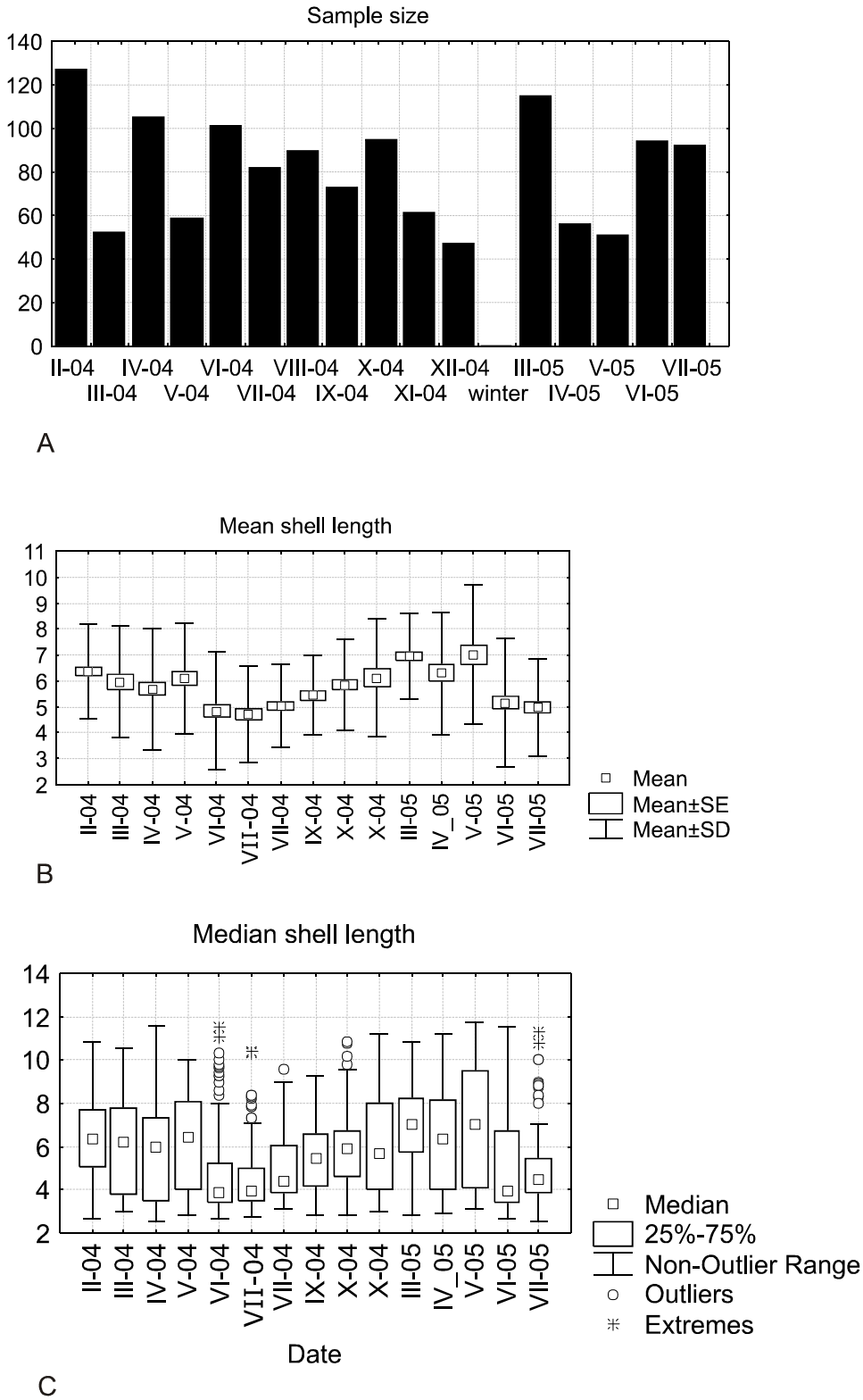

Fig 1 – A – Sample size, B,C – mean (B) and median (C) shell length in monthly samples

Figure 2

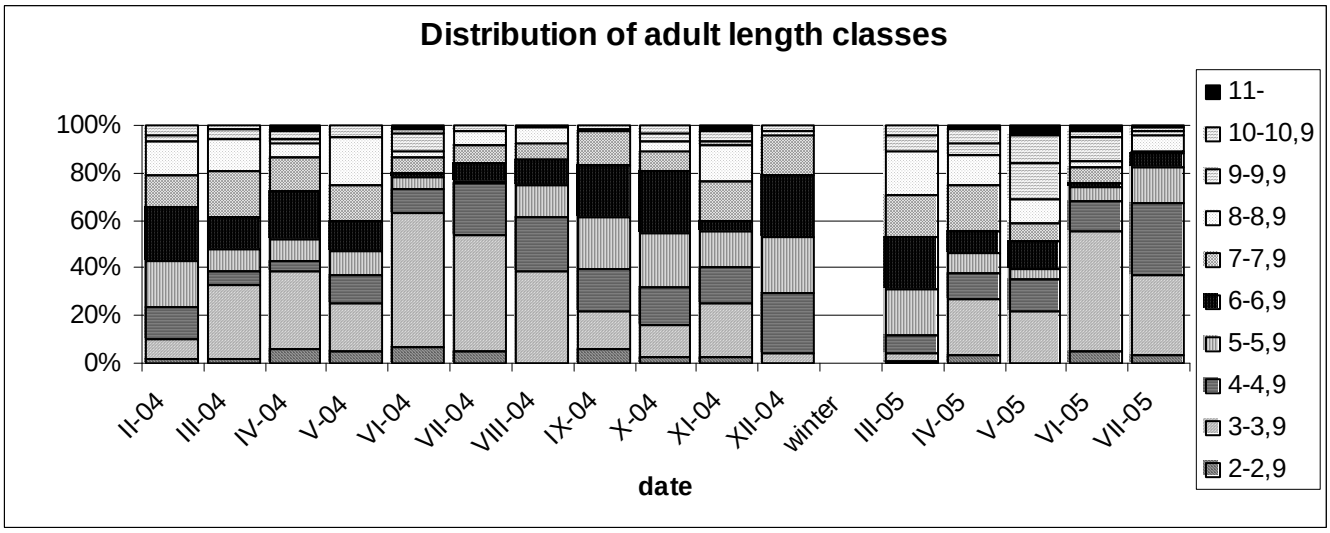

Fig 2 – Proportion of the adult length classes in monthly samples

Figure 3

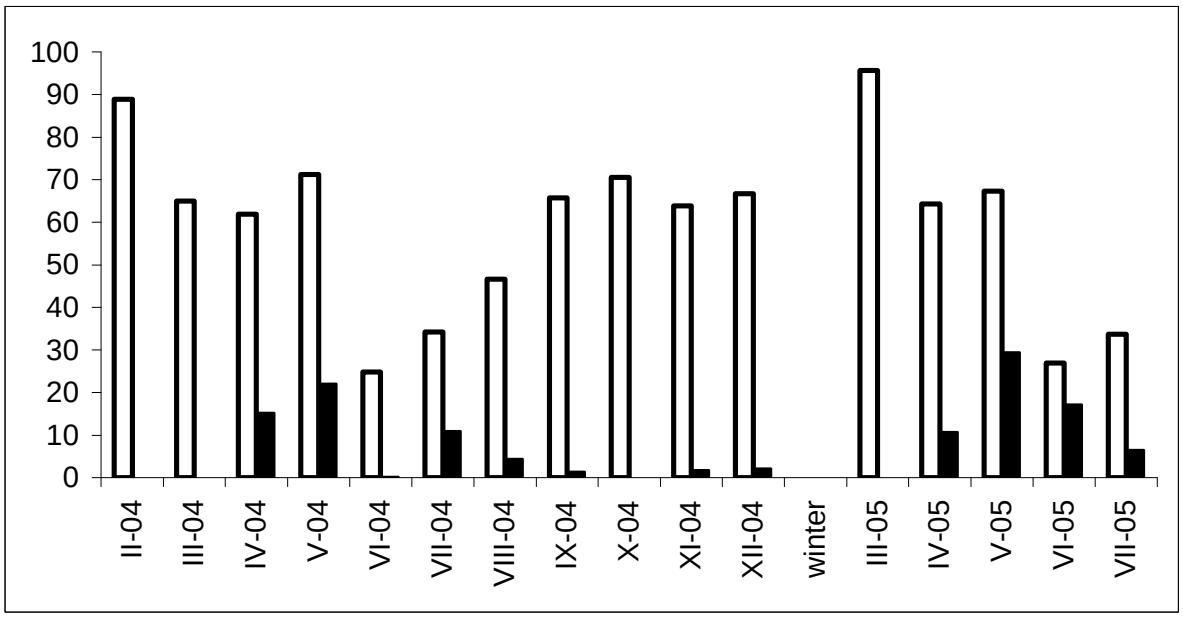

Fig 3 – Total percentage of gravid adults (bearing larvae of any developmental stage, white columns) and percentage of adults bearing EML (black columns) in monthly samples.

Figure 4

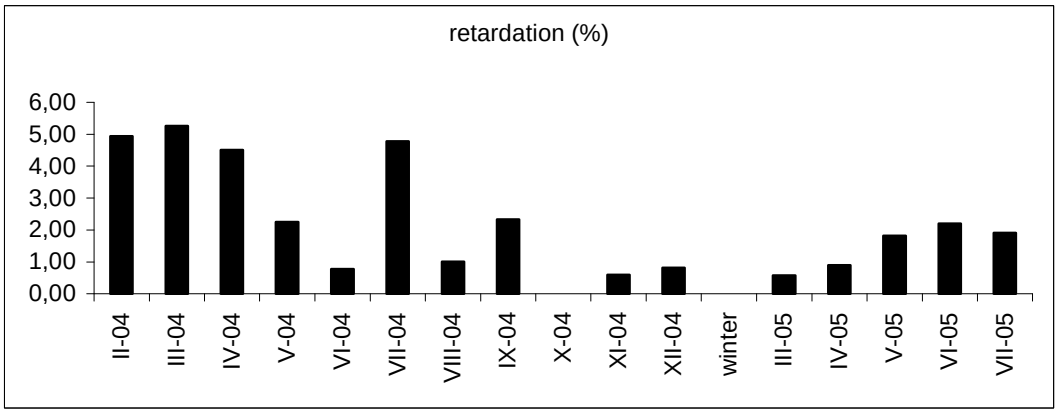

Fig 4 – Percentage of retarded larvae in monthly samples

Figure 5

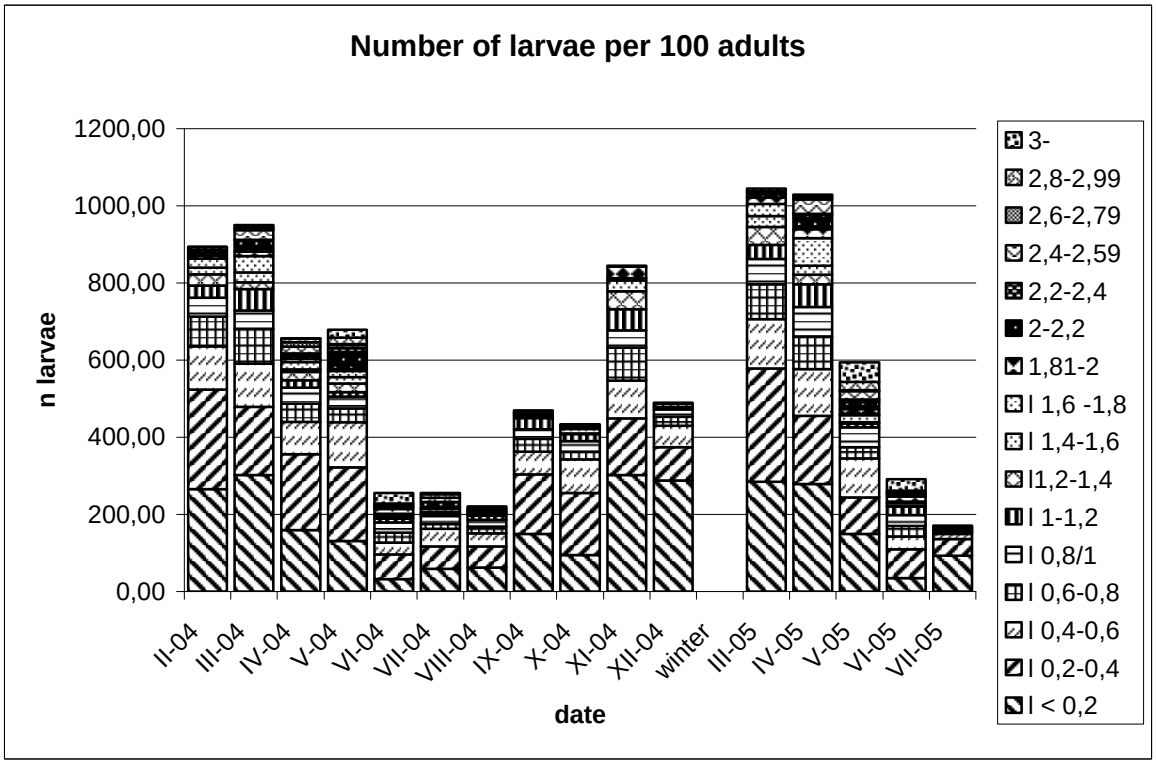

Fig 5 – Percentage of larval length classes

Figure 6

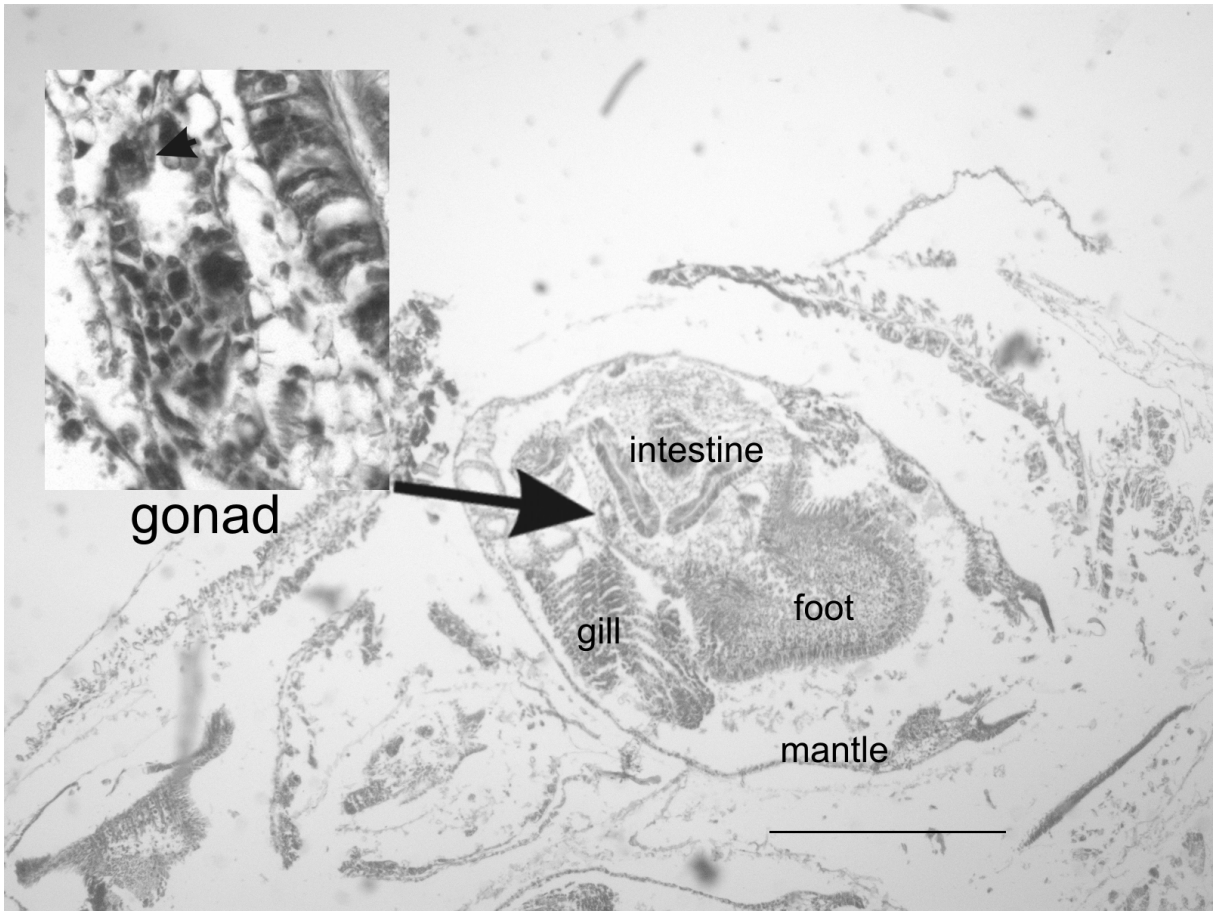

Fig 6 – Part of paraffin section through an adult *S .corneum* (shell length 8 mm, sampled in May 2004), showing a well-developed extramarsupial larva with oogonia (arrowhead) in the gonad (detailed view in the left upper corner). Stained with Masson´s triple stain, scale bar = 1 mm.

Chapter 4 – Kořínková, T. accepted (Malacologica Bohemoslovaca): Food utilisation in fingernail and pill clams.

Food utilisation in fingernail and pill clams

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Abstract

The contents of digestive systems in six freshwater bivalve species of the family Sphaeriidae were investigated. Microorganisms in the stomachs and intestines of the bivalves were the same as found suspended in the water, which implies none or very limited preingestive selection, at least of the organic particles. Most of the organisms (coccal algae, diatoms, flagellates, euglenophytes, Zygnematophycaea, monadoid algae, bacteria) probably pass through the digestive system unharmed, as they were found alive even in the alimentary bolus in the hindgut. Probably only a small proportion of them is digested, in particular the bacteria and monadoid algae, the abundance of which decreased especially following starvation. In starved animals, food particles were also present in the digestive diverticula, apparently in order to increase food utilisation.

Key words: Sphaeriidae, digestive system, filtration, ingestion, water microorganisms

Introduction

Eulamellibranchiate bivalves are typically filter –feeders. The suspension of potential food particles is pumped in through the inhalant opening or siphon. It also contains some indigestible elements, the proportion of which is regulated during filtration on the ctenidia and labial palps by the rejection of some heavier particles in the form of pseudofaeces. Some authors believed the particles in the suspension to be sorted only by physical forces without any active selection prior to entering the digestive canal (JORGENSEN 1996, WAY 1989). However, the rejection rate of inorganic particles has been found to depend on the concentration of suspended organic matter (HAWKINS et al. 1998, WONG & CHEUNG 1999, BAYNE et al. 1987), which is an argument in favour of the concurrent hypothesis supposing physiological regulation of food uptake (BAYNE 1998).

The incoming current then proceeds through the mouth to the oesophagus, which connects to the stomach, followed by an intestine. The latter can be divided into the midgut (the part containing typhlosoles and the crystalline style), the coiled intestine, and the hindgut, with the anus positioned near the posterior adductor.

In the stomach, further sorting takes place, facilitated by movements of epithelial cilia. The crystalline style helps mechanical sorting (and perhaps also disintegration) of the particles and produces enzymes (REID 1968, MORTON 1973). The style itself undergoes cyclic periods of abrasion and reformation (secretion of the crystalline style matter by epithelial cells), at least in intertidal bivalves (MORTON 1973). This implies the digestion is a discontinuos, cyclic process. The general mechanism of digestion as described below has been studied mostly in marine species, but it is with all probability similar in freshwater lamellibranchiates as well. Sorted food particles pass into the digestive diverticula, the blind tubules of which probably are involved in the secretion of enzymes (esterases and endopeptidases, REID 1968), absorption and intracellular digestion (OWEN 1955). Waste products are sent back to the intestine to join rejection currents from the sorting area.

Some inorganic particles that are not rejected as pseudofaeces and pass through the digestive system might facilitate the mechanical breakdown of food particles (NAVARRO et al. 1996). The organic particles found inside bivalve digestive systems have been identified as mostly diatoms, small dinoflagellates and naked flagellates (REID 1968). BOUGRIER et al. (1997) reported preferential ingestion of flagellates and rejection of diatoms in marine lamellibranchiates *Crassostrea gigas* and *Mythilus edulis*. Under laboratory conditions, bivalves have been fed with monoalgal diets (NAVARRO et al. 1996), mixtures of various microscopic algae, and plant detritus (CHARLES & NEWELL 1997).

The primarily freshwater clams of the family Sphaeriidae often dominate among benthic invertebrates of lotic and lentic ecosystems. Detailed anatomical and histological descriptions of their digestive tracts are given by, for instance, MONK (1928) or HOLOPAINEN & LOPEZ (1989). The mechanism of food intake in Sphariidae differs to some extent from that typical for the most bivalves (MITROPOLSKII 1966, HOLOPAINEN 1985, LOPEZ & HOLOPAINEN 1987). Sphaeriids do not maintain a direct contact with the water column. They rather burrow into the substrate and draw the incoming current into their mantle cavity by an active process, probably facilitated by a cilial groove on the foot (MITROPOLSKII 1966). Therefore, sphaeriid clams should be characterized as interstitial suspension-feeders (HOLOPAINEN & LOPEZ 1987) or deposit-feeders (WAY 1989) rather than filter-feeders. Filtration of food particles is likely to occur, too, but it might play a minor role. MITROPOLSKII (1966) has measured the filtration rate in *Sphaerium corneum* and stated that filter-feeding is in this case not sufficient to cover the energy needs. Also the studies of HORNBACH et al. (1984) and RAIKOW & HAMILTON (2001) have proven prevalence of deposit- over filter-feeding in sphaeriids.

Although the mechanism of food intake has been so widely studied, there is a lack of data on the food preferences of sphaeriid clams, but the range of potentional food sources would probably be as broad as in the case of marine species. LOPEZ & HOLOPAINEN (1987) suggest that interstitial bacteria, including saprophytic ones, form the main part of *Pisidium* diet, whereas larger *Pisidium* species and *Sphaerium* and *Musculium* feed mainly on phytoplankton. RAIKOW & HAMILTON (2001) hypothesized about preferential utilization of algae by *Sphaerium*. FOE & KNIGHT (1986) succeeded in feeding *Corbicula fluminea* (family Corbiculidae, a group related to Sphaeriidae) for 30 days in laboratory culture with an artificial diet including unicellular green algae of the genus *Ankistrodesmus*. However, as the authors themselves have pointed out, the clams were losing weight during the experiment, suggesting that the monoalgal diet was not an optimal resource of food. The only information on diet of Sphaeriidae in rearing comes from MACKIE & FLIPPANCE (1983), who reported the utilisation of coccal algae and leaf litter as nourishment of *Musculium securis* grown in laboratory conditions.

Material and methods

The clams were sampled during summer 2009 from three sites in the Czech Republic: the Vltava river in Prague (shallow littoral zone with sandy bottom, 50° 05´ N, 14° 25´ E, species: *Sphaerium corneum, S. rivicola, Pisidium supinum),* the Rokytka stream in Prague (small stream, width ca. 1m, depth ca 0.5m, with sandy bottom, 50° 04´ N, 14° 36´ E, species: *S. corneum, P. casertanum*) and a small temporary drain in the Poodří Protected Landscape Area (maximum depth 20 cm, muddy bottom, surface overgrown by vegetation, coordinates 49° 42´ N, 18° 05´ E, species: *S. nucleus, P. milium*). The specimens were originally collected to dissect out the gonads, gills and larval stages for karyological experiments. The digestive tracts thus remained intact and could be used for wet mounts in a drop of water. The narcotisation (by immersion into water saturated with carbon dioxide) and dissections of the animals were carried out immediately after collection (three specimens of each species from a collection site) or after a certain period of starvation in clear water (three groups of animals

killed after 24, 48, 72 hours; each group contained three specimens of each species, the specimens being of different age and size classes). The parts of the digestive tracts (oesophagus, stomach, digestive diverticula, intestine) were treated separately. The exterior of each organ was carefully rinsed in distilled water, then a longitudinal section was led through the wall of the organ, the contents of which were extracted into a drop of water using a preparation needle. The preparations were covered with a cover slip and immediately observed under a light microscope in normal light or under differential phase contrast at ×100 magnification. All organisms in the visual field were counted; the mean from 10 randomly chosen, non-overlapping visual fields was used to estimate the total number of each taxon in the sample. Approximately 0.5 l water samples and water-leaches of the sediments from natural habitats were, after centrifugation to a convenient concentration, also inspected for microorganisms. These were primarily classified according to their morphological characteristics and mechanical properties, resulting in some algae being determined to the genus or species level, whereas other organisms could only be identified as “coccal bacteria” or “spirochaetes”.

Results

No oesophagus of any species investigated contained microorganisms. Microorganisms found in the digestive tract and the water sample were principally the same (Fig. 1): coccal bacteria, which dominated in number (estimate 10^4-10^5 in adult *S. corneum*), spirochetes, monadoid algae, diatoms (*Fragilaria*, *Navicula*, *Pinularia*, *Tabelaria*), green algae (*Scenedesmus*, *Coelastrum*, *Eudorina* sp., *Pediastrum simplex*, *P. duplex*, *Volvox* sp.), Zygnematophycae (*Closterium*, *Zygnema*, *Cosmarium*), ciliophores (in particular Oligotrichea), euglenophytes.

The diversity and abundance of organisms in the digestive tract of freshly captured clams was very similar to that of the surrounding water. Some specimens of *Pisidium* differed in containing smaller number of green algae, ciliophores and diatoms (Fig 1 c) than *Sphaerium* did. Apart of that, no striking differences in stomach and intestine content were found between clams representing different species or age classes. Epithelia of the stomach and intestine retained some of their physiological activity even a few hours after the narcotisation and death of the animal: their cilia were still beating in the wet mounts. In spite of this, most of the microorganisms were apparently alive and intact even in the accumulated material in the hindgut: green algae retained their cytoplasm colour and many of the organisms that are normally able to locomote (some bacteria, euglenophytes, ciliophores) were actively moving. In clams following 48 and 72 h of starvation, the stomach contained less green algae, but these were found intact in the intestine, implying that their tough cell walls allow them to pass through the digestive tract unharmed. The proportion of bacteria in the intestine decreased markedly after starvation, and they, together with some coccal algae were also the only microorganisms found in the digestive diverticula – the organ where part of the digestive process takes place.

Discussion

This study presents a brief overview on occurrence and abundance of selected groups of microorganisms in the digestive tracts of the sphaeriid freshwater clams (Bivalvia: Sphaeriidae).

The oesophagus was always depleted of any particles which implies very rapid passage of the ingested suspension through this muscular organ. The following parts of the digestive tract contained the same microorganisms as the water and sediments from the locality. Most of them passed unchanged through the digestive system rather than being utilised. Probably it is mainly the inorganic particles that are rejected in the form of

pseudofaeces, on account of their size and weight, but a detailed analysis of the pseudofaeces should be made to confirm this supposition.

JORGENSEN (1996) suggested that the particles are captured by the gill apparatus mainly by means of a fluid mechanical process, characterized by a low Reynolds number (i.e. laminary flow). The process would thus be governed by the fluid velocity and by physical properties of the particles rather than by their digestibility or nutritive value. The present observations are in accordance with this expectation .

Most of the microorganisms occurring suspended in the water are probably indigestible thanks to their cell wall or – in the case of diatoms – resistant frustula. The persistence of food particles in the stomach after three days of starvation can be explained 1) by a slow passage of the solid particles through digestive tract even in normal conditions, and 2) by stopping through flow and enhancing utilisation as the concentration of suspended particles in the water decreases. The decrease in abundance of bacteria and some algae in the stomach and their presence in the digestive diverticula after starvation would imply they are probably digested preferentially (or exclusively). Another, though less probable explanation for the distribution pattern of bacteria would be that some of them are symbionts facilitating digestion of some nutrients, and that these symbionts die out when the nutrient income stops.

No apparent interspecific differences have been found with respect to the utilization of particular groups of microorganisms. The small abundance or absence of green algae, ciliophores and diatoms in some specimens of *Pisidium* might support the suggestion of HOLOPAINEN (1985) that smaller *Pisidium* species feed mainly on bacteria whereas larger S*phaerium* species utilize phytoplankton. However, it might also be a mere consequence of the fact that clams with smaller body size are not able to ingest larger particles. In the specimens investigated by myself, the stomach and intestine contents were mostly composed of the same microorganisms as found in the surrounding water. It was not possible to test for differences between the composition of the water suspension and that of the sediment leaches. Therefore, it cannot be estimated what proportion of food particles came from filtration of the water current or from deposit-feeding, respectively.

Some elegant laboratory experiments with monocultural diets containing selected microorganisms (e.g. only monadoid algae, only euglenophytes, diatoms etc.) would provide a better knowledge about the possibility of the digestion of the non-preferred components in the absence of the preferred ones. The design of the reported experiment also did not allow evaluating ingestion and digestion of organic detritus, the particles of which could be hardly quantified by means of light microscopy and might nevertheless be one of the preferentially digested food components.

Conclusion

The present study confirmed the absence of any special sorting mechanism which would allow freshwater sphaeriid clams (Bivalvia: Sphaeriidae) to ingest preferentially the utilisable food particles. Microorganisms of suitable size that are found suspended in the water pass into the bivalves´ stomach. Some of them are then disintegrated by the action of the crystalline style and digested, but the most continue their passage through the digestive tract without any disruption and often even stay viable. The results suggest small detritus particles (not detectable by the method used) or bacteria as the main source of nutrients for the small freshwater bivalves.

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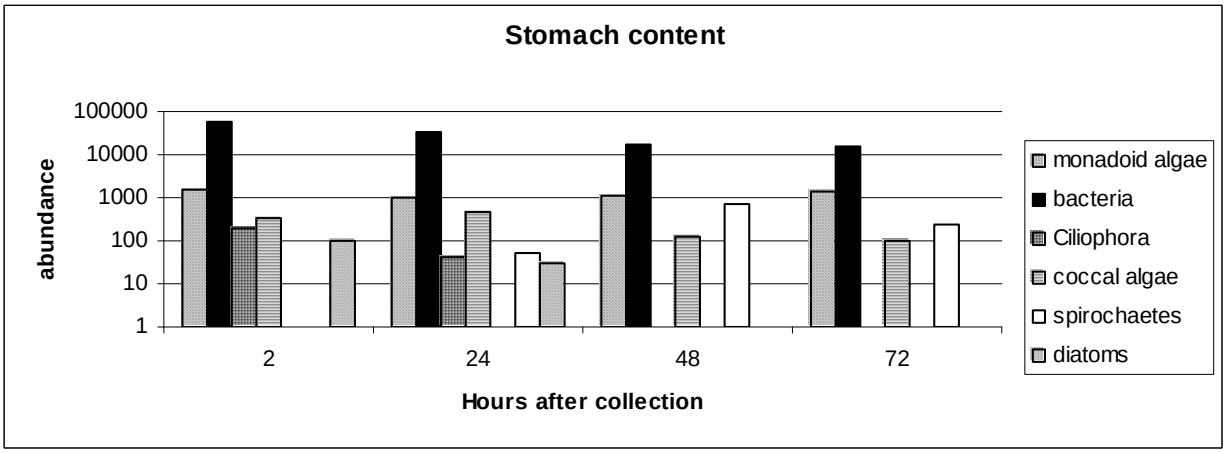
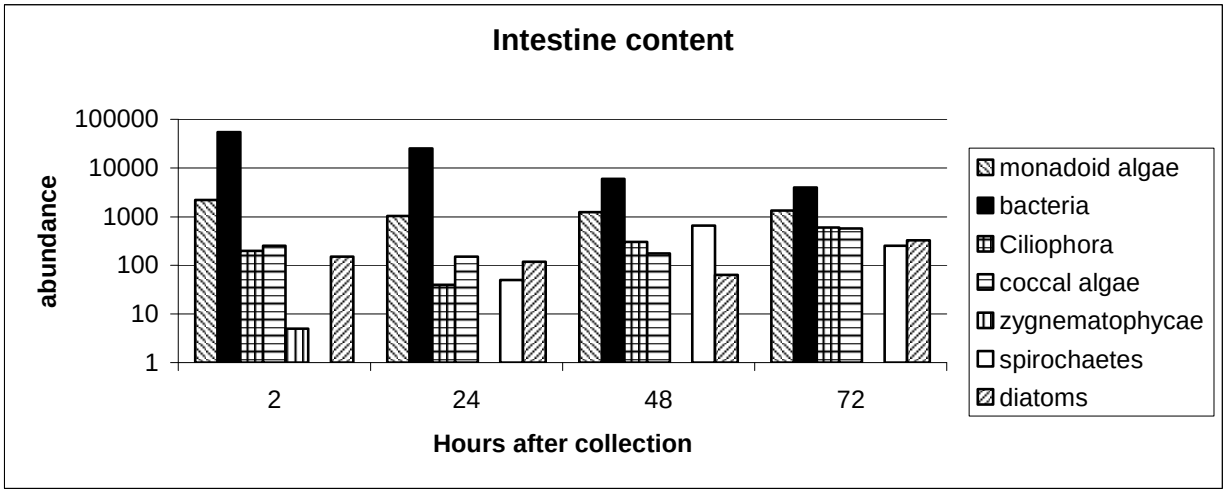
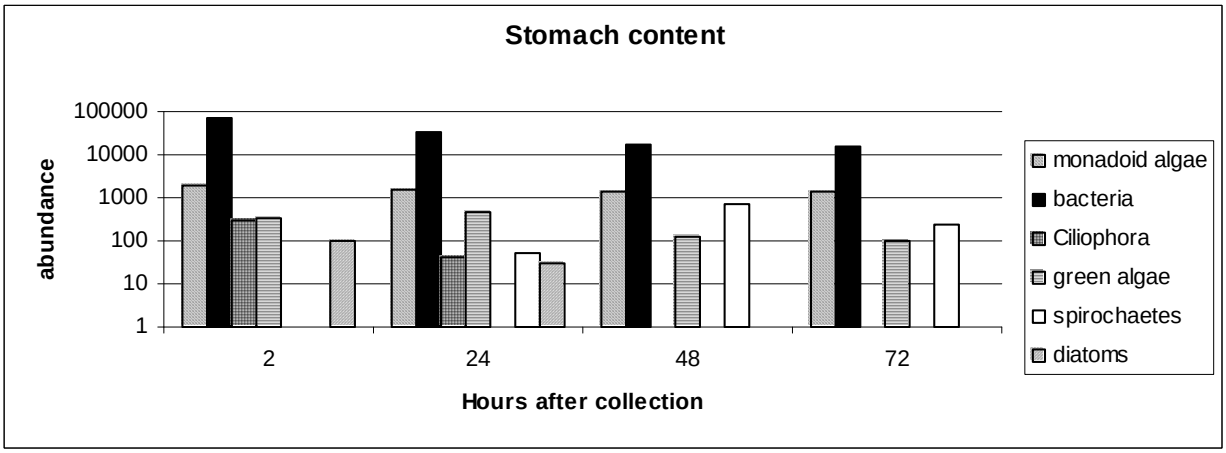
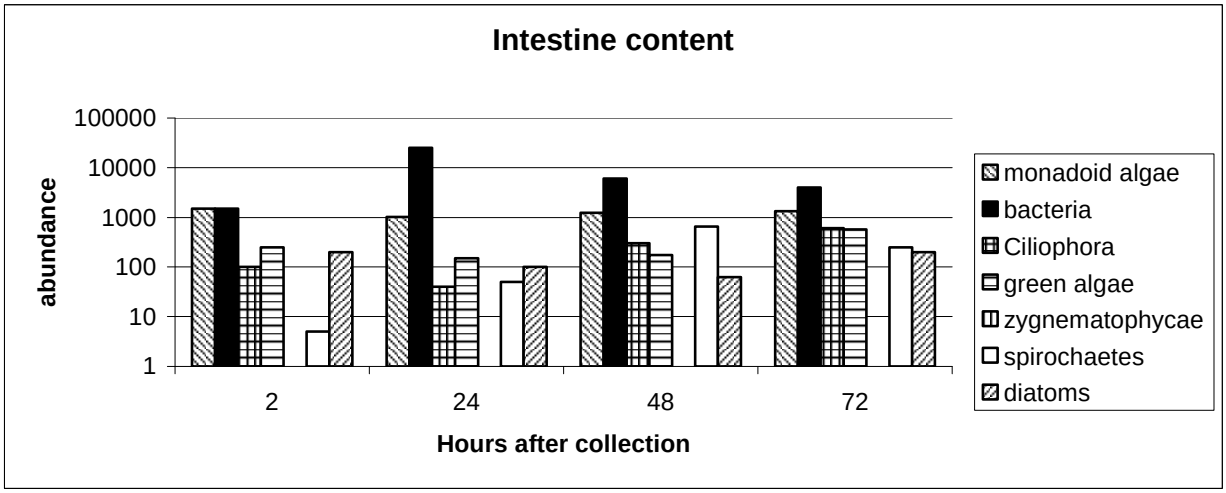
Fig. 1. An example of changes in the stomach and intestine content of sphaeriid clams from Vltava river during starvation: mean abundances of the most important groups of microorganisms counted from the three specimens dissected in each time period. A) *Sphaerium corneum, B) S. rivicola, C) Pisidium supinum.* from Vltava river, D – Abundance of microorganisms in 1 l of water from the collection site (Vltava river). Note the logarithmic scale of abundance.





A

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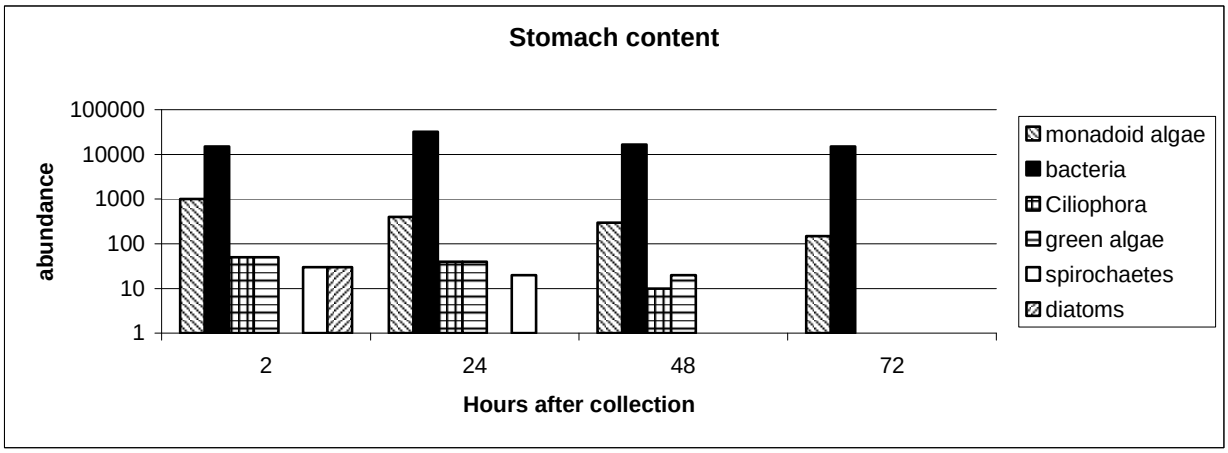
bacteria
Ciliophora
green algae
zygnematophycae
spirochaetes
diatoms

B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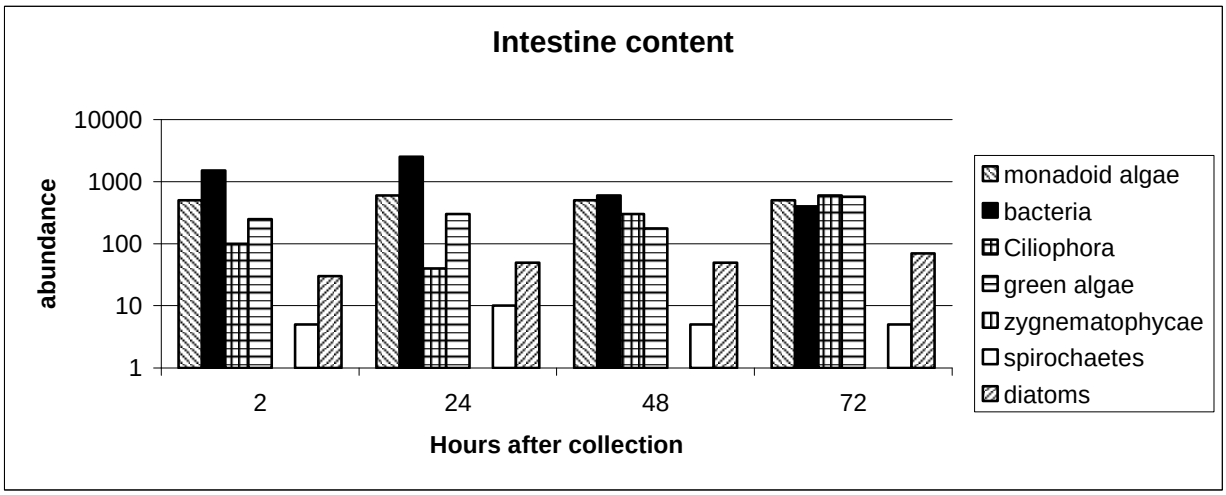
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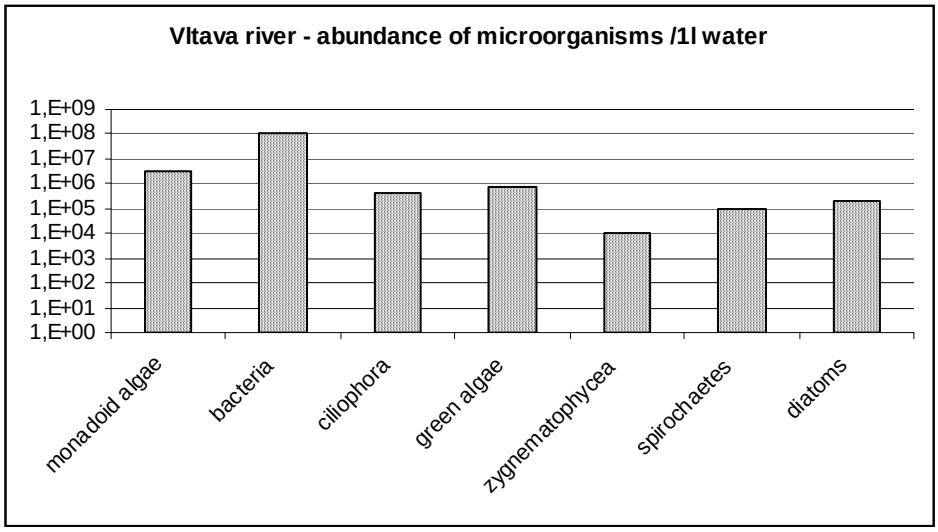
Vltava river - abundance of microorganisms /1l water
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1,E+03
1,E+02
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monadoid algae
bacteria
ciliophora
green algae
zygnematophycea
spirochaetes
diatoms

D

Conclusion

In the present thesis, selected representatives of the family Sphaeriidae (genera *Sphaerium*, *Pisidium* and *Musculium*) have been investigated with respect to their cytogenetics as well as some anatomical, physiological and biological aspects, namely that of reproduction and food uptake.

Altogether, all three presently recognised *Sphaerium* species available in the Czech Republic were used in course of this study, including *S. nucleus*, the occurrence of which in the area has recently been proven (Kořínková 2006 a,b, Kořínková et al. 2008). In particular, the most abundant species *S. corneum* was represented by most (eight) populations, which were examined cytogenetically, one of them was further used for life-history studies and two for investigations of feeding strategies. The involvement of *S. nucleus*, the closest sibling species of *S. corneum*, into this study (two *S. nucleus* populations from the Czech Republic were examined cytogenetically) was especially feasible with respect to the previous investigations on the relationship of the two taxa (Kořínková 2006 a,b, Kořínková et al. in prep.). 16 S data from Lithuanian (Petkevičiūtė et al. 2006) and Czech (Kořínková et al. in prep.) populations indicate, on the basis of 16S sequence data from more *S. nucleus* populations, the existence of more cryptic "*S. nucleus*" lineages.

Karyotype analyses failed to find any reliable characters for distinction between *S. corneum* and *S. nucleus*. The differences are rather at interpopulational level. They concern mostly the morphology (meta- or submetacentric) of some chromosome pairs and the number of B chromosomes. Nevertheless, *S. rivicola*, which has also been examined cytogenetically in course of this study (Chapter 1), possesses a karyotype completely different from those of *S. corneum* and *S. nucleus*. As discussed in Chapter 1, the high chromosome number of *S. rivicola* (ca 240 versus 30 in the two remaining species) and the prevalence of acrocentric chromosomes over the biarmed ones raise the question if the species is congeneric with the remaining two.

Data on chromosome numbers and morphology were also obtained for nine *Pisidium* species and for *Musculium lacustre*. All of them exhibited similar karyotype characteristics as *S. rivicola* – high chromosome counts (over 100) and a high proportion of acrocentrics. This finding has lead to the decision to measure and compare DNA contents in selected species.

The DNA contents of species with 30 chromosomes were found similar or even slightly higher than those of the species exhibiting high chromosome numbers. None of the DNA contents seems to be a multiple of another. The results contradict a hypothesis that the recent species with a low chromosome number (*Sphaerium corneum* and *S. nucleus*) were diploid ancestors of the polyploid species. Also the difference in chromosome size and morphology between the two groups indicate that the karyotypes with high chromosome numbers have undergone several rounds of rearrangements (by fissions, fusions, and translocations). Such changes, together with elimination of the genome size, are typically involved in the process of diploidization occurring in palaeopolyploids. I agree with Lee (2001) that it is difficult to estimate the basic chromosome number of Sphaeriidae. I would suggest that the ancestral karyotype was similar to that of the recent diploid lasaeid and corbiculid clams (i.e. 2n 36 or 38, mixture of biarmed and monoarmed chromosomes). Such a karyotype might have produced by centric fusions the karyotypes with 30 biarmed chromosomes, and the karyotypes with high chromosome numbers would be derived by polyploidization followed by further chromosomal rearrangements. It is possible to hypothesize about the analogy between the real-time polyploidization processes occurring in recent representatives of Lasaeidae and Corbiculidae and those that predated the evolution of Sphaeriidae.

An important contribution to the discussion on “diploidization” of the presumably polyploid karyotypes of Sphaeriidae were the observations of meiosis in *S. rivicola* (2n=240) and *P. henslowanum* (2n=190). The metaphase plates at first meiotic division consisted with all probability prevailingly or entirely of bivalents. Chiasmata could be observed at least on the larger bivalents. This is in contrast to the achiasmatic course of meiosis in *S. corneum* and *S. nucleus*. As achiasmatic meiosis is generally considered a derived feature (White 1973), the recent observations are a further argument supporting an ancient divergence of the lineages representing the recent species with low chromosome numbers and the paleopolyploids. Nevertheless, the chiasma formation in *S. rivicola* seemed to be delayed up to diakinesis stage (cryptochiasmatic meiosis). This implies that the evolution towards achiasmatic meiotic division was parallel in both lineages, and it was almost completed in one of them whereas the other exhibits a mode of meiotic division transitional between the chiasmatic and achiasmatic one. Analogical differences in the course of meiotic division at the family level have been found e.g. in the spider family Dipluridae (Král et al. submitted, Král et al. in prep.)

In course of this PhD. project another interesting aspect of meiosis in *Sphaerium* has been found – the first meiotic division is probably achiasmatic both in the spermatogonial and oogonial part of the gonad. As discussed in Chapter 2, such a pattern is quite unusual. Also the “diffuse stage” of chromatin decondensation probably occurs both in male and female meiosis of the hermaphroditic organism. It must be noted that separation of the testicular and ovarial part is extremely difficult in freshly killed animals – usually it is even a problem to find the gonad at all. The evidence for the meiotic sequence being the same in both germlines is based on histological sections. These have shown (Woods, 1931; Heard, 1965, Chapter 3 of this thesis) that corresponding developmental stages of spermatogonia/spermatocytes and oogonia/oocytes are present in the gonad simultaneously. The meiotic divisions take place from spring to autumn, usually with two distinct peaks, and gamete maturation occurs not only throughout this period, but probably – though with a lower intensity – also in the remaining part of the year (data are missing for a short period in winter).

The combination of monthly sampling and morphometric measurements with dissections, histological sectioning and chromosome preparations, as described in more detail in Chapter 3, has brought a complex insight into the life history characteristics of one *S. corneum* population. The population was chosen due to its high population density, accessibility and also due to the fluctuation of physical parameters (water level, oxygen content, biomass). Comparison with published life-history data on *Sphaerium* species suggest that some of the observed features (overlap of generations, continuous release of broods between the main peaks of reproduction) probably represent a specific adaptation to the habitat. On the other hand, some characteristics have been found in all investigated populations including the one presented in this thesis. These aspects are probably common to the whole family, or at least to the subfamily Sphaeriinae: the simultaneous hermaphroditism, continuous gametogenesis with certain peaks and accordingly the multiple fertilization and incubation of more broods differing in their developmental stage. Histological sections have also proven the already reported precocious maturation (occurrence of gametogenesis in larvae incubated within parents´ gills – Thiel 1928, Heard 1977), but as quite a rare phenomenon.

The fluctuations in the seasonal activity and reproduction are for sure influenced by the actual offer of food particles. Comparison of the stomach contents with the microorganisms in the water of the habitat (Chapter 4) supports the hypothesis that Sphaeriidae take in all suspended particles of suitable size. Only a small proportion of them is really digested, as inferred from the presence of many intact, usually even viable, algae and bacteria in the terminal part of the intestine. The experimental layout did not enable to follow

the utilization of detritus particles which, being potentially more digestible than whole microorganisms, might possibly account for much of the energy intake. The utilisation of detritus would also be an explanation for findings of some active individuals even in winter months, when the ice cover has just thawed and the concentration of algae and bacteria must have been very low. Of the microorganisms, some bacteria are also likely to be digestible for the sphaeriids, as their abundance in the intestine decreased in clams after starvation. But, as noted in Chapter 4, care must be taken to distinction between bacteria as food particles or as possibly parasitic/comensal/symbiotic species. If the decrease in abundance of bacteria really resulted from their utilisation as food particles, it would be a good example of “postingestive regulation” (in contrast to “preingestive selection”studied e.g. by Bougrier et al. 1997) of food uptake. But a proof and detailed analysis of such a mechanism is far beyond the scope of this thesis.

To conclude, the combination of interconnected thematic issues included in this thesis has yielded a complex view of some biological features of sphaeriid clams. The cytogenetical data would moreover contribute to the discussion on phenomena like palaeopolyploidy, achiasmatic meiosis and structure of behaviour of B chromosomes. In this respect, the content of presented thesis has to some extent exceeded its original scope.

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(list of references cited in the Introduction and Conclusions section. See also References in the particular chapters)

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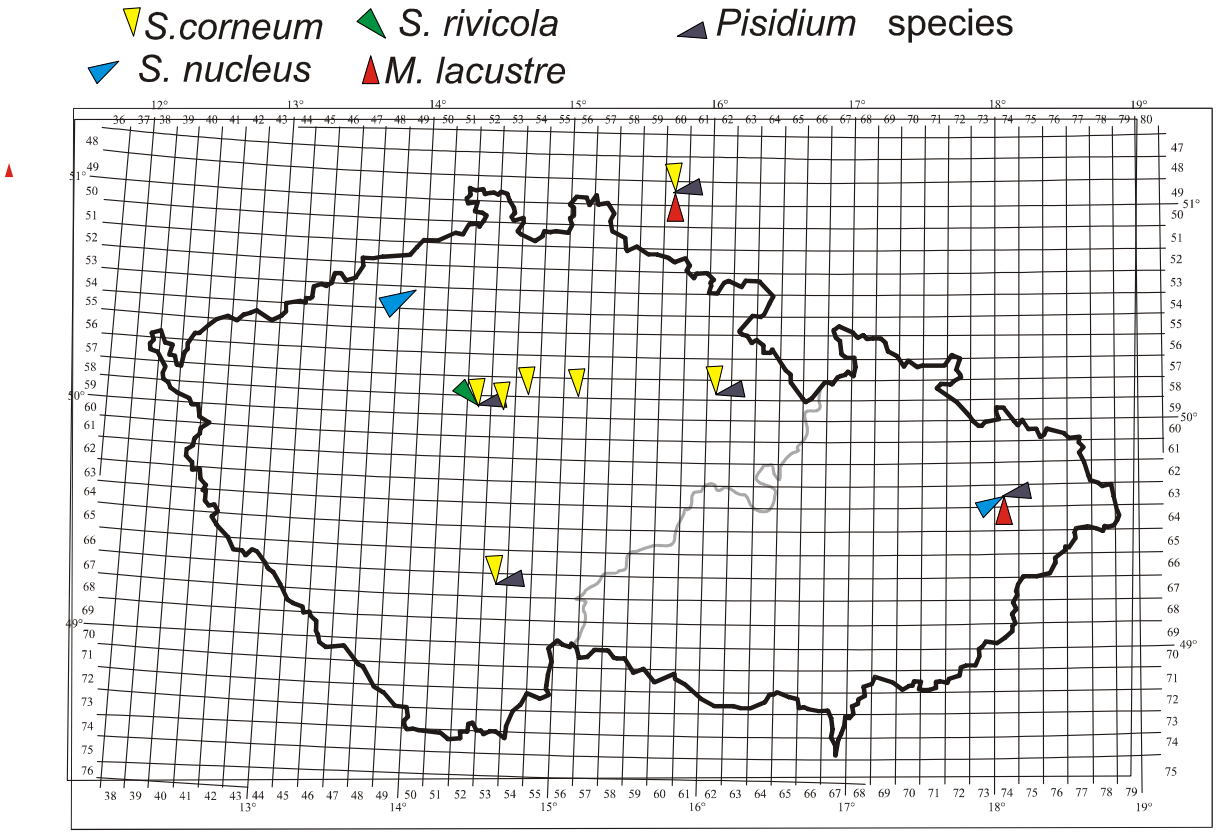
Supplements

Map of collection sites (populations used for cytogenetical studies)

(Populations from Central Poland are not figured. The tips of the arrowheads point into the corresponding square of the grid map.)

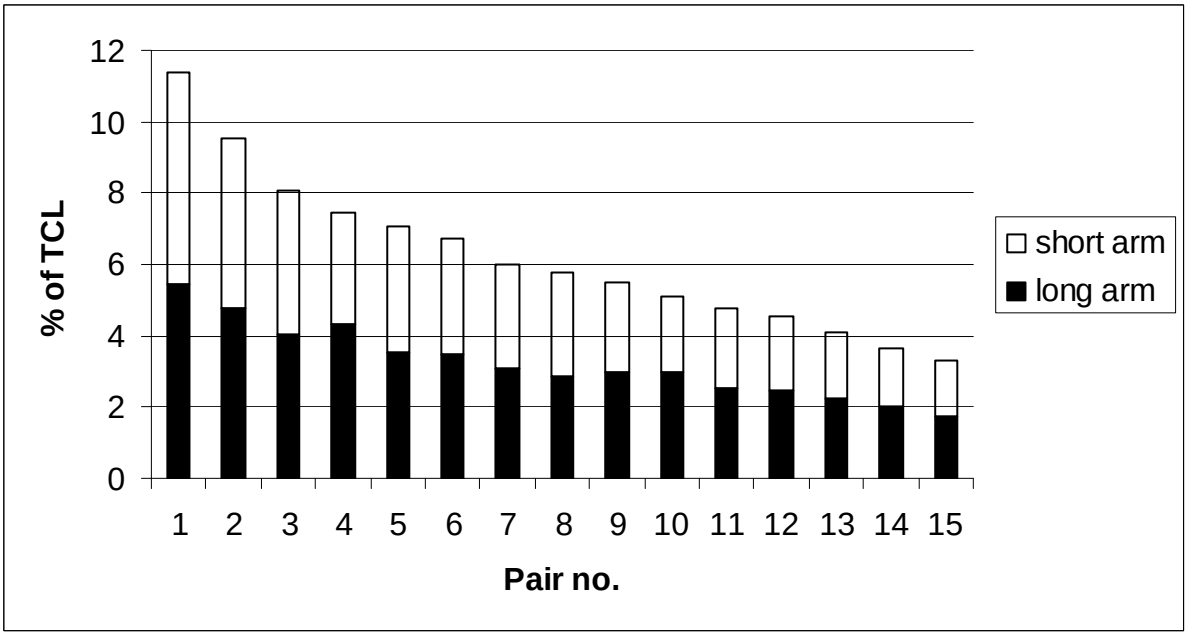


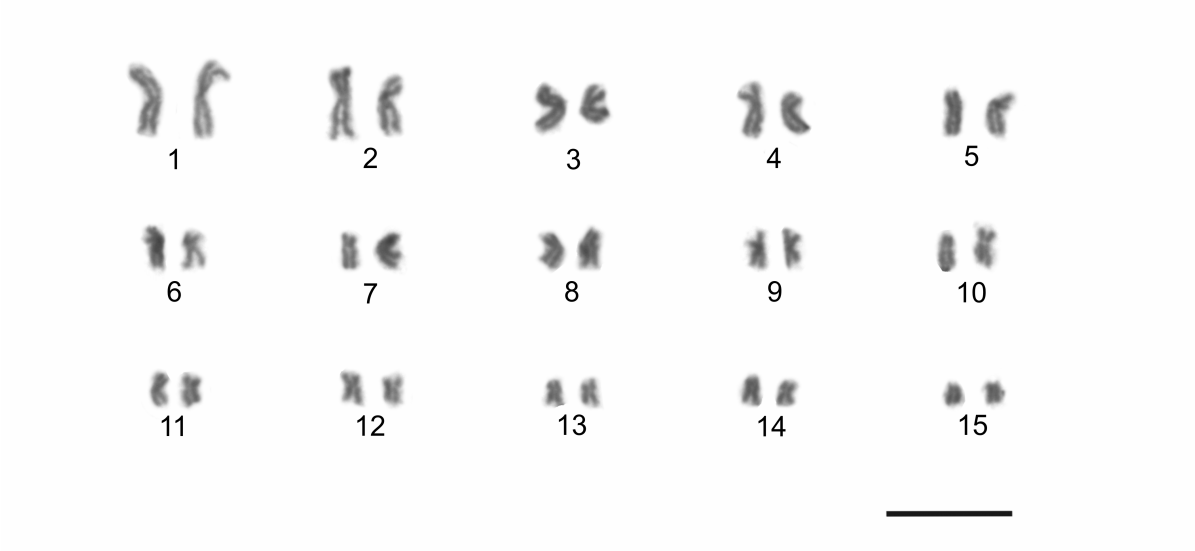
Idiograms and karyotypes of *S. corneum* and *S. nucleus* populations

Each population is represented by an idiogram and karyotype of one particular late mitotic metaphase, selected from the plates that were used for calculation of the average. Scale bar=10 μm.

***S. corneum*, Bechyňský potok stream**

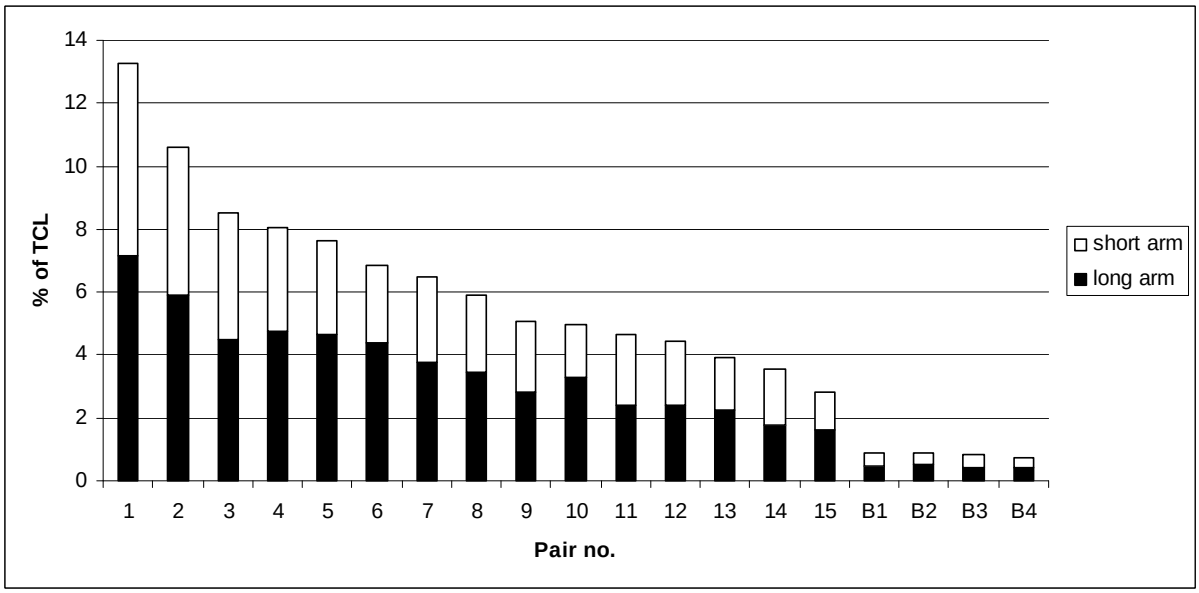
Specimen without B chromosomes

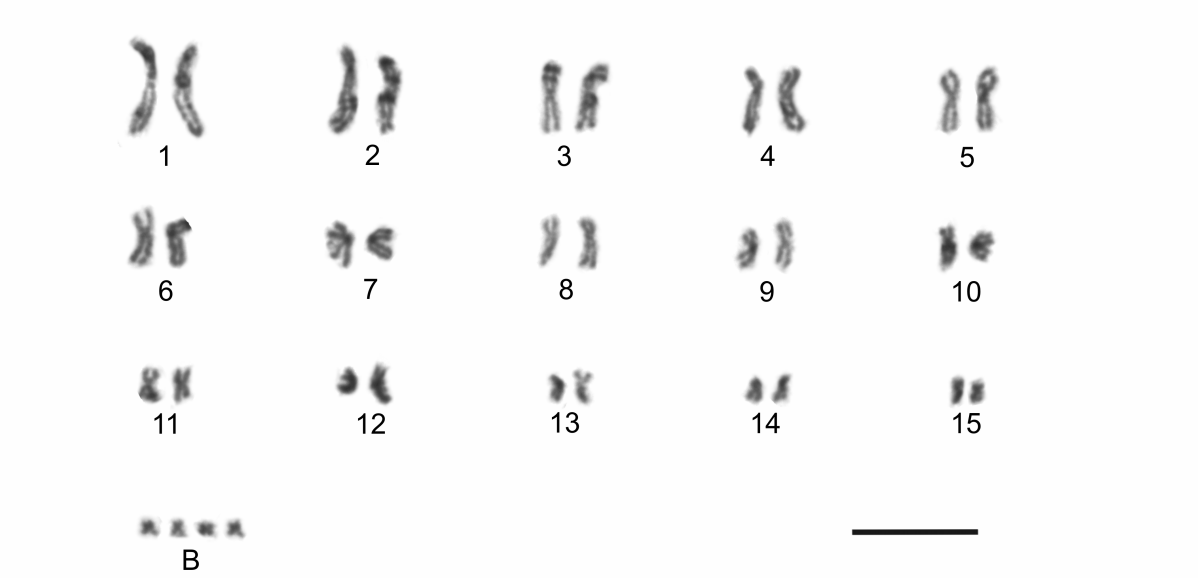




***S. corneum*, pools near Čelákovice-Sedlčánky**

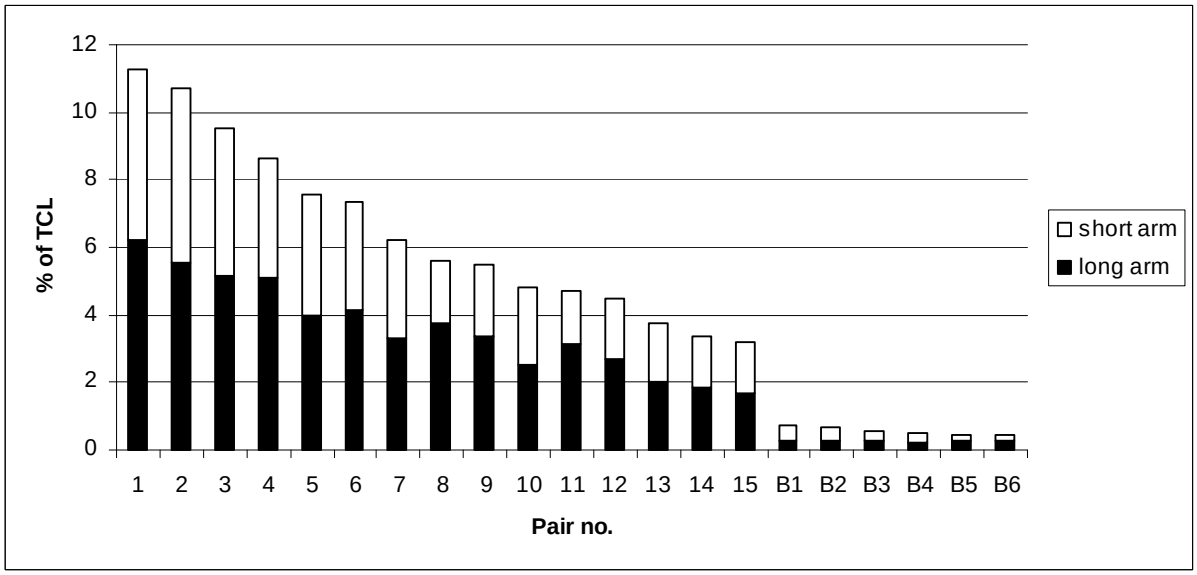
Specimen with four B chromosomes

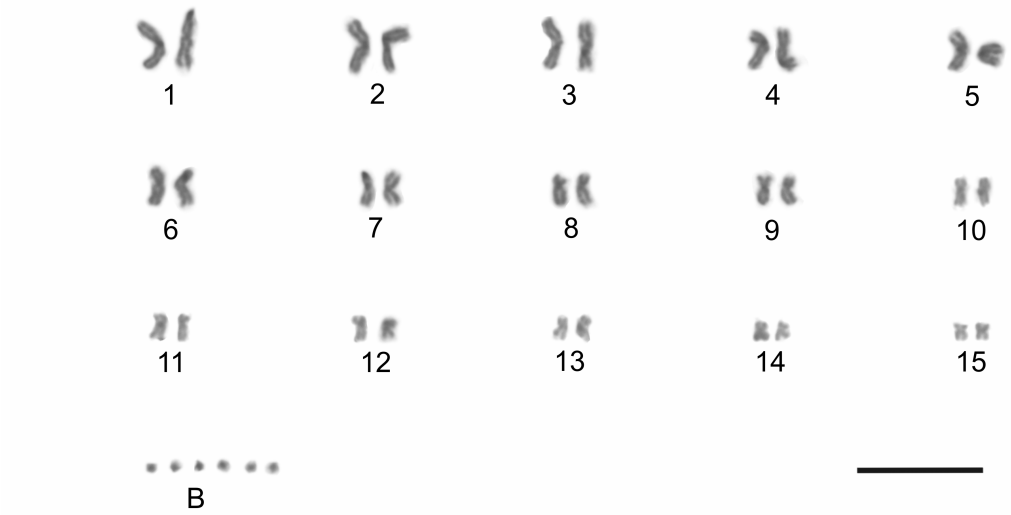




***S. corneum*, Gorlitz – Weinlache**

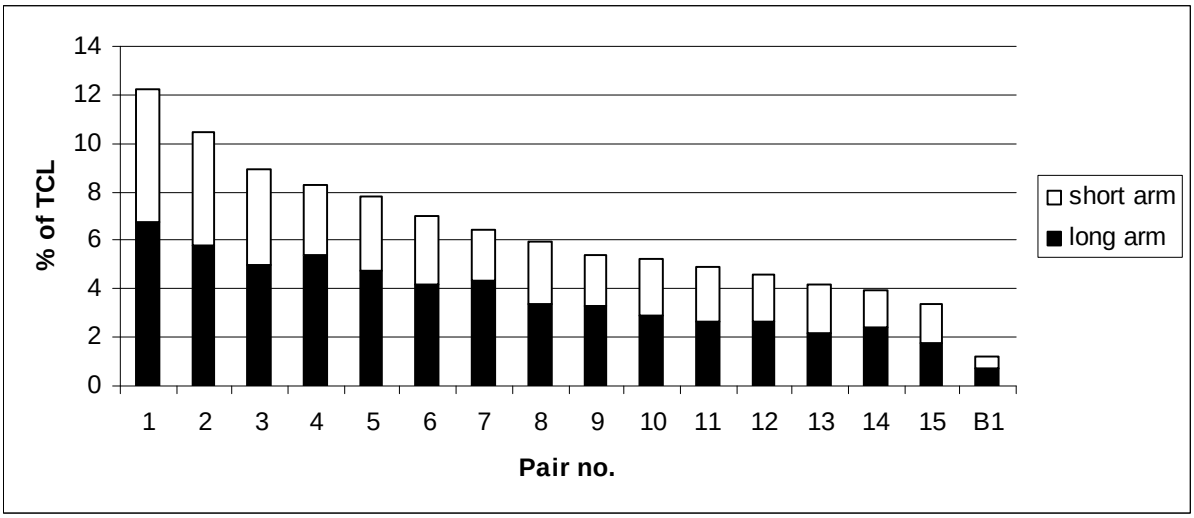
Specimen with six B chromosomes

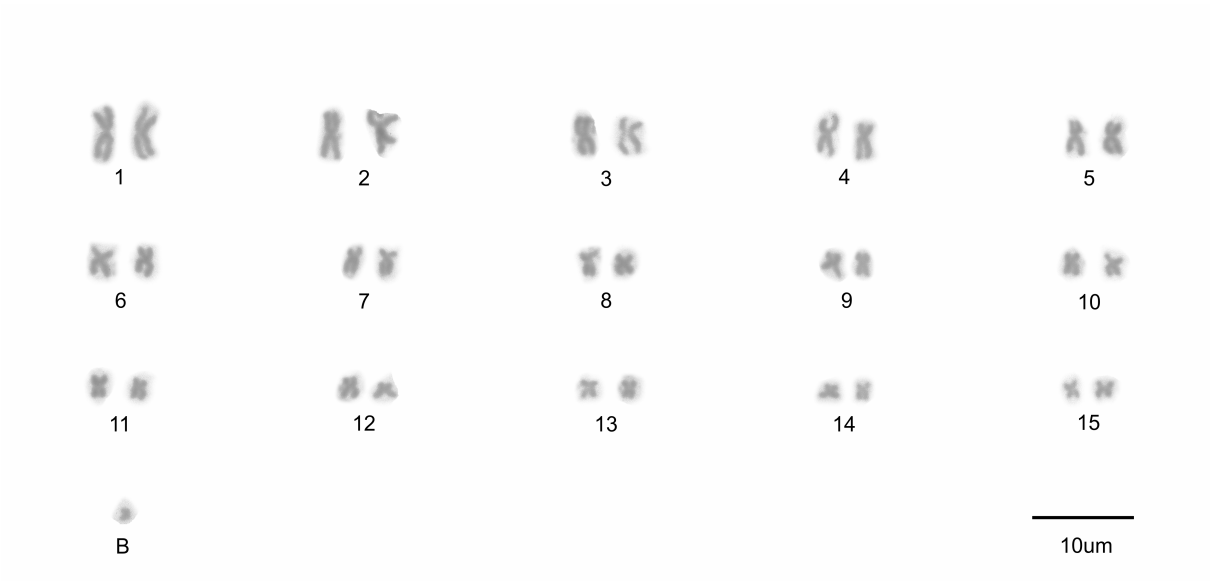




***S. corneum*, Maškův mlýn**

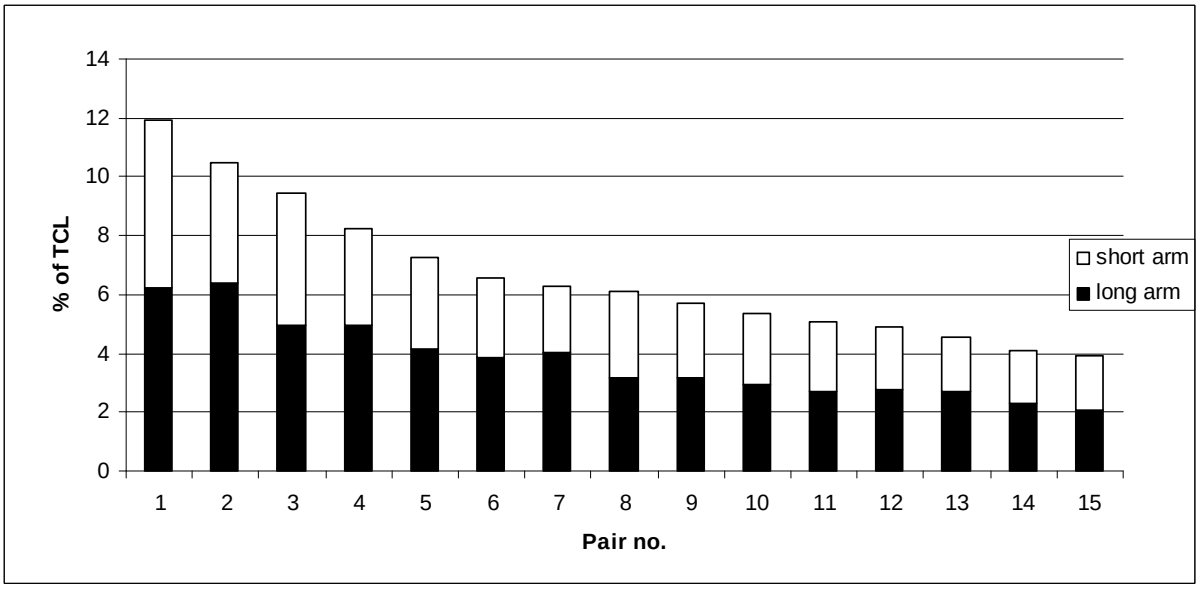
Specimen with one B chromosome

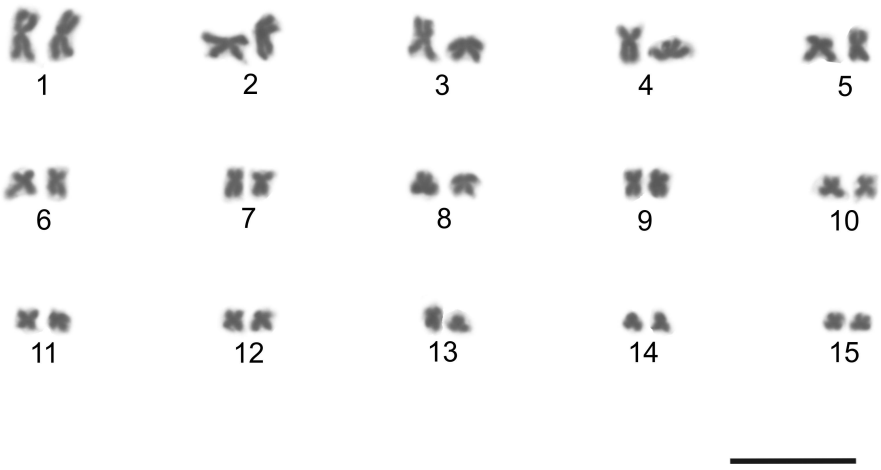




***S. corneum*, Rokytka stream**

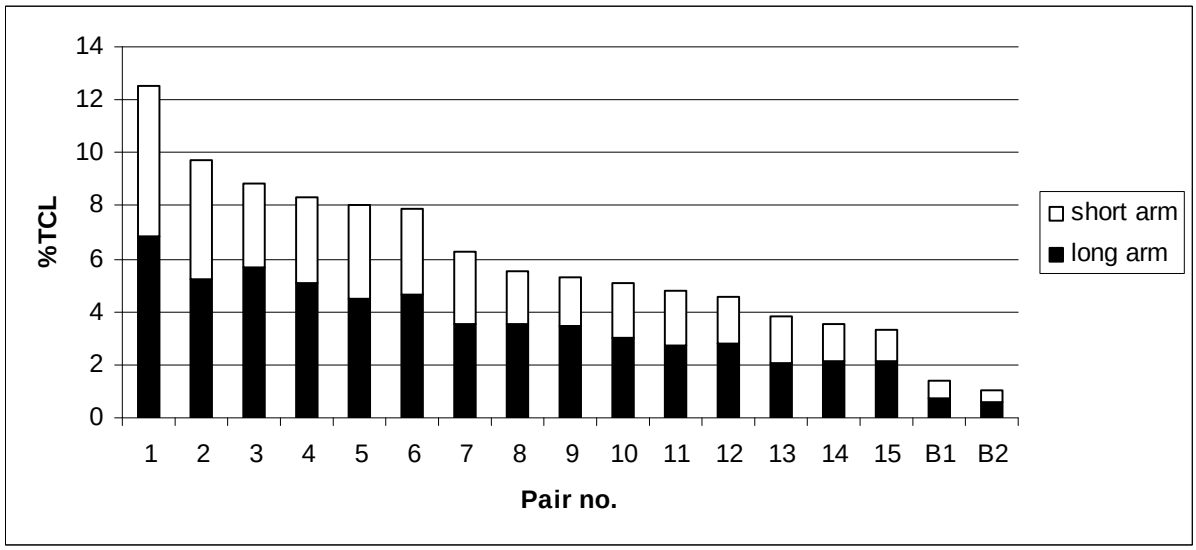
Specimen without B chromosomes

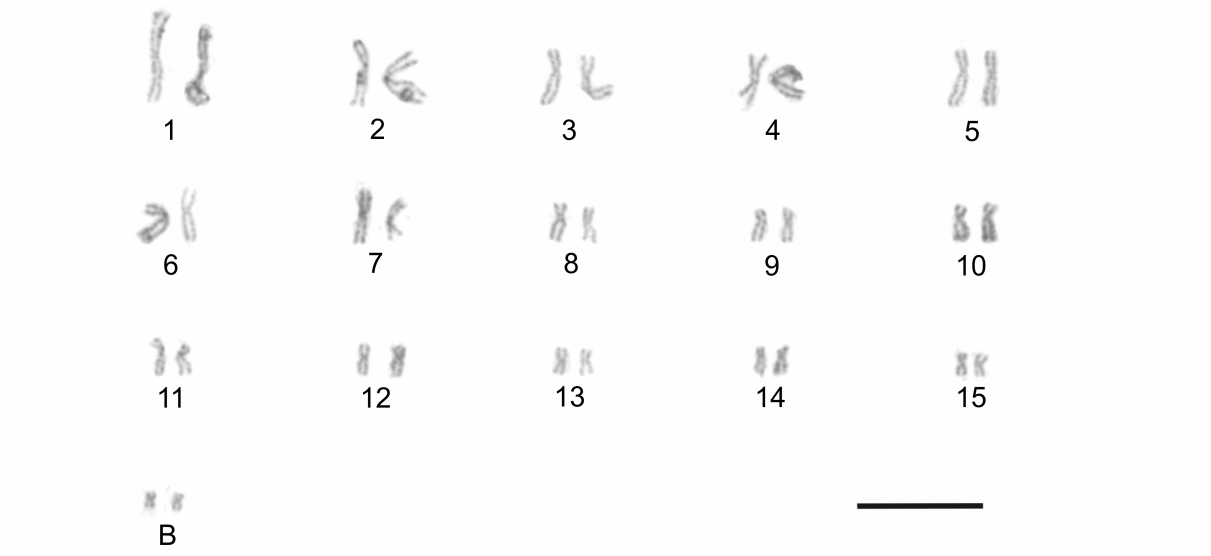




***S. corneum*, Sánský kanál drain**

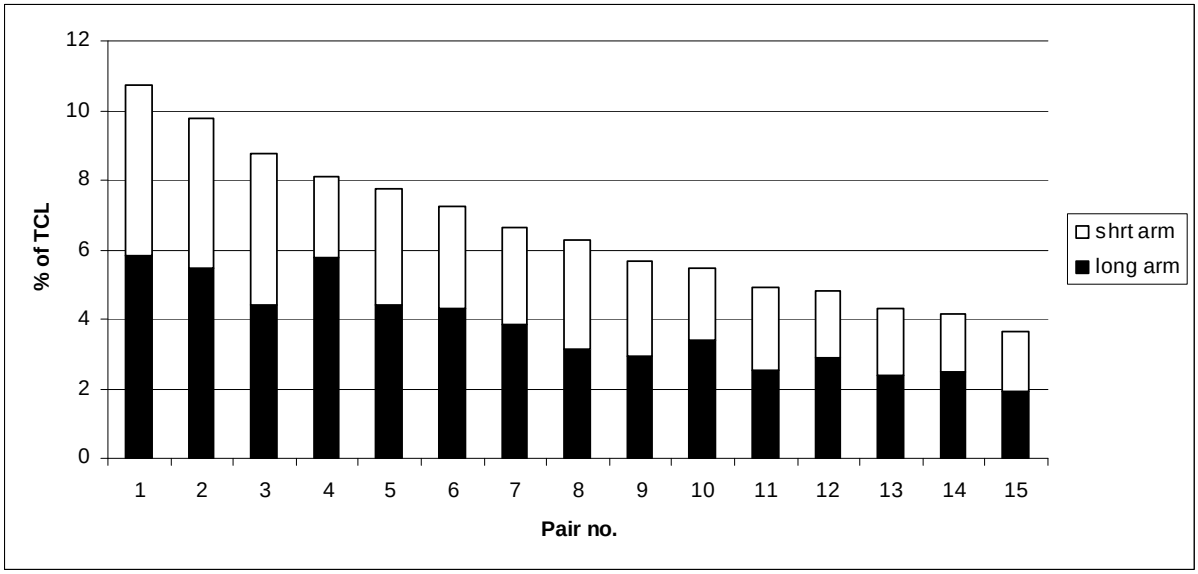
Specimen with two B chromosomes

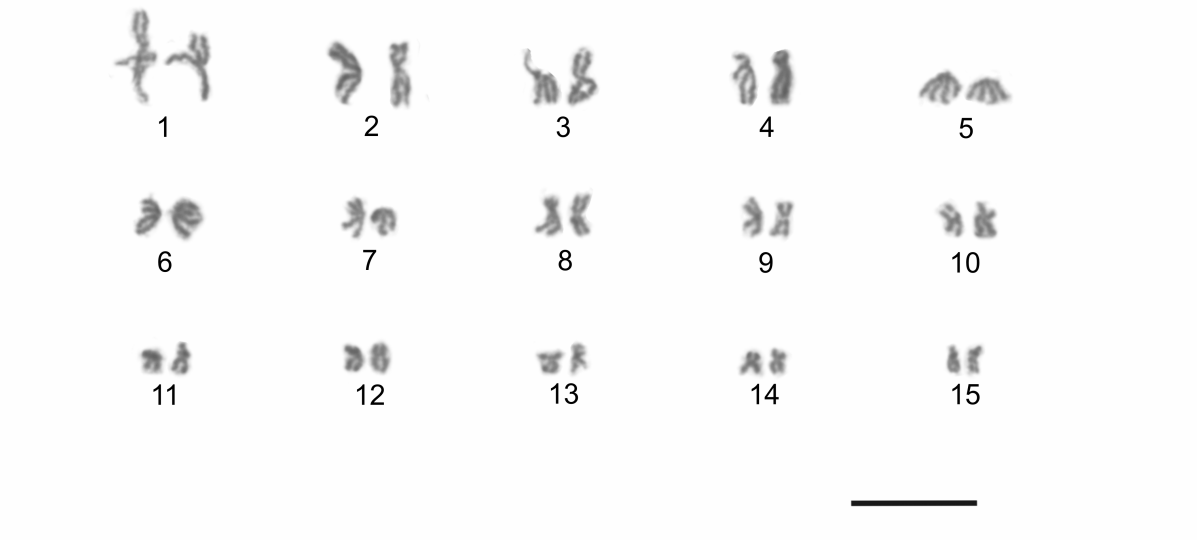




***S. corneum*, Weißer Schöps stream**

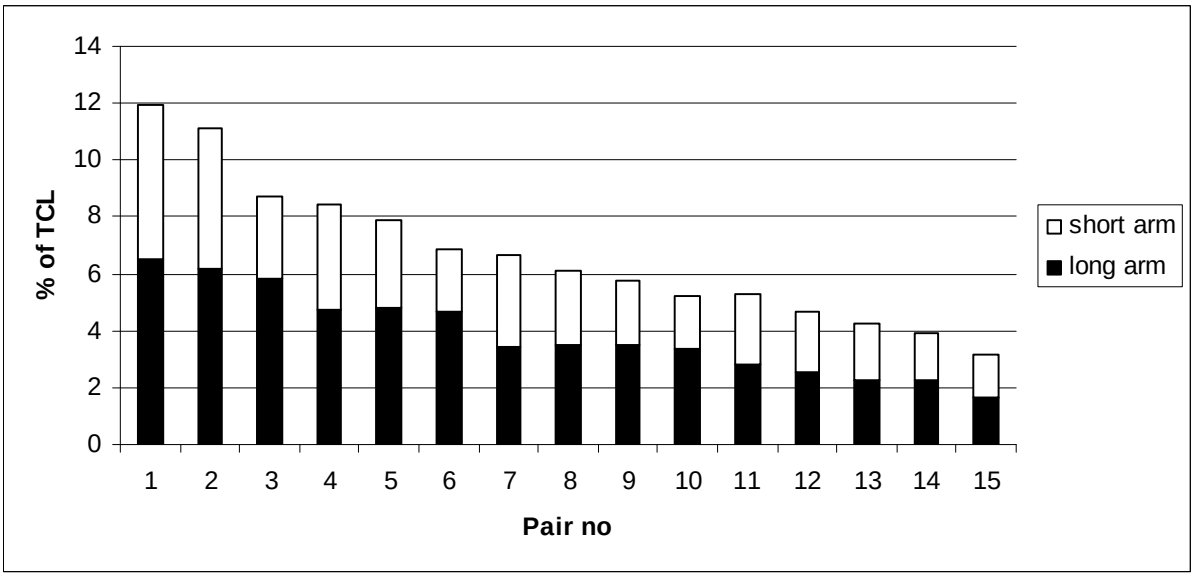
Specimen without B chromosomes

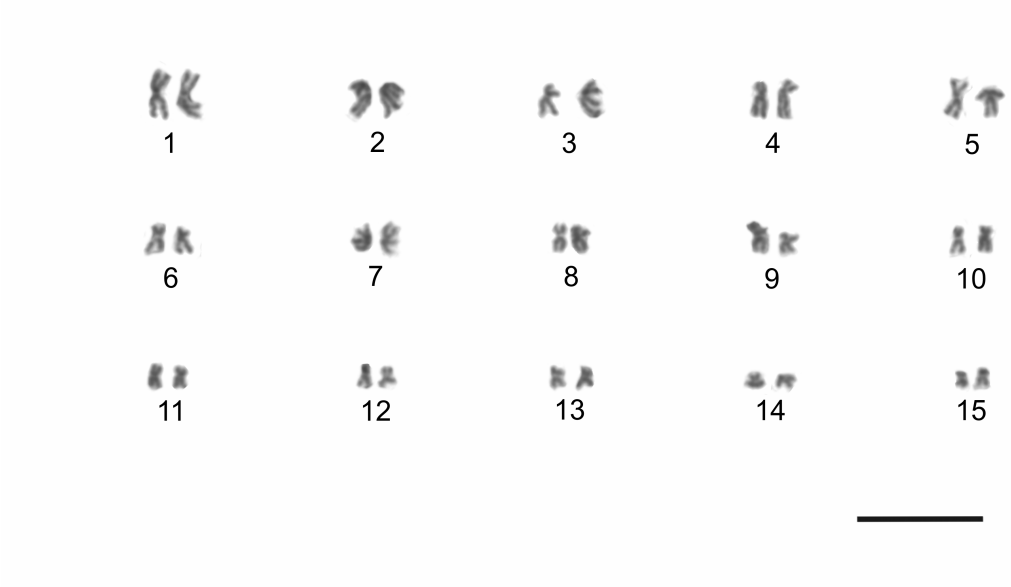




***S. corneum*, Vltava river**

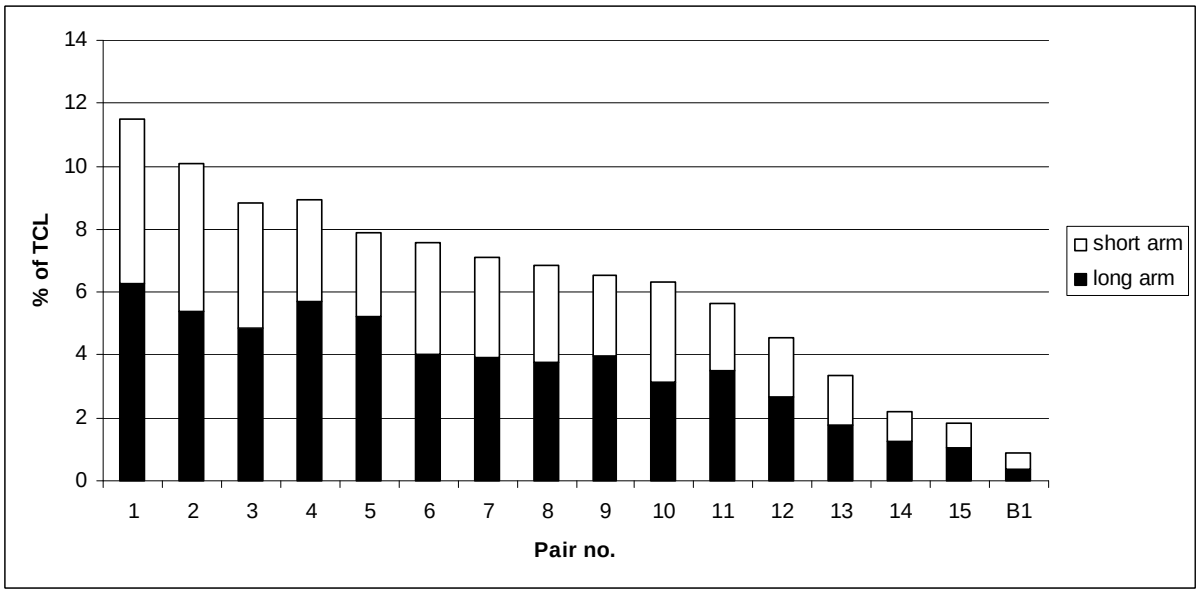
No B chromosomes found in the population

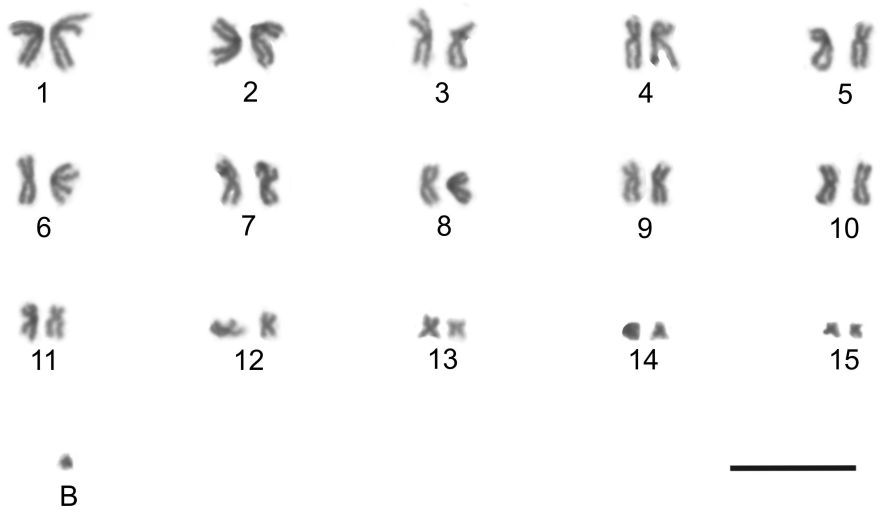




***S. nucleus*, Březina Nature Reserve**

Specimen with one B chromosome





S. nucleus, Poodří Protected Landscape Area

Specimen with four B chromosomes