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ON SEXUAL DIMORPHISM OF KARYOTYPES OF *VIVIPARUS VIVIPARUS* AND *V. CONTECTUS* (GASTROPODA, VIVIPARIDAE)

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On Sexual Dimorphism of Karyotypes of *Viviparus viviparus* and *V. conlectus* (Gastropoda, Viviparidae). Andriychuk, T. V., Garbar, A. V. — Karyotype analysis of males and females of two Viviparid species *Viviparus viviparus* Linnaeus, 1758 and *V. conlectus* (Millet, 1813), shows differences in their karyotypes in the absence of heteromorphic pairs of chromosomes. This indicates an epigamous mechanism of sex determination.

Key words: Viviparidae, karyotypes, sex chromosomes, sex determination, sex ratio.

К половому диморфизму кариотипов *Viviparus viviparus* и *V. conlectus* (Gastropoda, Viviparidae). Андрийчук Т. В., Гарбар А. В. — Анализ кариотипов самцов и самок двух видов живородок — *Viviparus viviparus* Linnaeus, 1758 и *V. conlectus* (Millet, 1813) показывает различия в их кариотипах при отсутствии гетероморфных пар хромосом. Это указывает на эпигамный механизм определения пола.

Ключевые слова: Viviparidae, кариотипы, половые хромосомы, определение пола, соотношение полов.

Introduction

Studies of aquatic life strategies under deteriorating environmental conditions in the water bodies of Ukraine raises the question of the reasons why some species fall into a state of depression, while others remain relatively stable. The main factors are environmental. Nevertheless, at the forefront of pivotal importance is the plasticity species, their ability to adapt to the changing environment, maintain an adequate level of feeding success. Changes affecting the conditions and forms of reproduction play far not the last role. An example can be the disruption of the normal sexual process on the way towards agamous reproduction and parthenogenesis. The latter circumstance is very important for a number of invertebrate groups that have weakly formed chromosomal sex determination mechanisms. Hence, there is a possibility of the reversion of sex, occurrence of hermaphroditism and spontaneous parthenogenesis, which all very likely can lead to a reduced level of reproduction.

In this regard, freshwater mollusks of the family Viviparidae are of interest. For example, the American representatives of the genus *Campeloma* Rafinesque, 1819 show a wide distribution of obligate and facultative parthenogenesis (Johnson, Leefe, 1989). As for the European representatives of the genus *Viviparus* Montfort, 1810 evidence of parthenogenesis has not yet been gained, although the sex ratio in the Ukrainian populations is far from an equilibrium and is characterized by a sharp deficit of males, and in some cases they are even entirely absent (Levina, 1992). A special analysis of sex ratios in populations of two ecologically closely related species *V. viviparus* (Linnaeus, 1758) and *V. conlectus* (Millet, 1813) showed that in depleted water bodies of Ukraine the populations of *V. conlectus* consist on an average rate of only 15 % of males, whereas in populations of the more or less thriving *V. viviparus* this figure reaches 25 % (Mezhzherin, Andriychuk, 2013). In addition, sexual dimorphism concerning traits of *V. conlectus* are manifested in body size, whereas the differences between males and females in *V. viviparus* are well expressed by the proportions of the shell.

All this gives evidence to believe that one of the reasons for such a sharp decline in the number of *V. conlectus* may be a partial transition towards agamous reproduction, which is not as effective as amphimixis. The gastropods are in the initial stage of the formation of bisexual mechanisms. This group is featured by both hermaphroditism and dioecism (Minichev, Starobogatov, 1971). However, for the family Viviparidae bisexuality is a rule, whereas unisexuality is an exception (Anistratenko, Anistratenko, 2001).

Due to the ambiguity of the sexual structure of the populations of viviparids, a question arises to what exactly determines dioecism amongst these mollusks: epigamous non-genetic factors or syngamous chromo-

somal mechanisms of sex determination? It may be that these mechanisms are combined, resulting in the prevailing influence of poor environmental conditions, whereas chromosomal mechanisms take over under more favorable circumstances. The available data do not give unambiguous answer to this question. According to Baršienė et al. (2000), a number of European species of the genus *Viviparus* have a ZW-mechanism of sex determination, but in the species *V. viviparus* and *V. contectus* heteromorphic pairs of chromosomes are absent. This result contradicts the data of Pavlyuchenkova (1997), which indicate the presence of a heteromorphic XY chromosome pair. An in-depth study of the karyotypic variability of males and females of *V. viviparus* and *V. contectus* in a comparative perspective, involving rigorous statistical approaches is needed.

Material and methods

To avoid a geographical bias, viviparids for the karyological study of the two species were collected from a restricted area in Zhytomyr Region of Ukraine. Mollusks were collected by hand at the period of their sexual activity (May–August).

Due to differences in the mitotic activity of the two species, the analyzed number of metaphase plates in *V. viviparus* turned out to be about twice larger than for *V. contectus*. Karyological slides of satisfactory quality were obtained from 42 individuals of *Viviparus viviparus* (21 ♂ and 21 ♀) from the following localities: directly from the Teteriv River (11 individuals), a pond near the village of Vysoka Pich (4 individuals), River Guyva (25 individuals) and a pond nearby the settlement of Sonyachne (2 specimens). The number of individuals of *V. contectus*, for which suitable slides were obtained, was significantly less (9 ♂ and 9 ♀): Korosten, River Uzh (3 individuals), the vicinity of the village Vysoka Pich (9 individuals), and a pond nearby the village of Reya (6 individuals).

Chromosome preparations were made using the air-dry method, treating the snails beforehand by colchicine (Poberezhniy, 1979). Young individuals for 16–16.30 hours were kept in a 0.02 % colchicine solution. Tissue of gonads was used to study the chromosome sets. After removing this tissue, it was crushed and kept for 15–20 minutes in distilled water for hypotonization. The material was then fixed in a mixture of ethanol and glacial acetic acid (3 : 1). Preparations were produced using the print method (Baršienė et al., 2000). The cell suspension by drop was placed on slides using a capillary pipette. Preparations were dried and stained for 20–25 minutes at 4–8 % solution of azure-eosin using a phosphate buffer (pH = 6.8) solution (according to Romanovsky).

The analysis of micro-preparations was performed under the microscope ULAB HU–B2 (camera magnification 100×10). For further studies metaphase plates with satisfactory placement of chromosomes and a roughly same level of spiralization were selected. We measured the length of the chromosomes, the length of the longer and shorter arms. Based on these measurements, we calculated the relative length of chromosomes (LR), the centromeric index (CI). The software package STATISTICA 6.0 was used for the statistical analysis of the data.

Results and discussion

The data on the number of chromosomes on the whole is consistent with other reports considering the karyotype of these species (Pavlyuchenkova, 1997; Baršienė et al., 2000). Individuals of *V. viviparus* have 9 pairs of chromosomes, and those of *V. contectus* have 7 pairs (fig. 1, 2). As for the chromosomal formula, there are minor differences compared to the published data. Judging from the measurements of the chromosomes (table 1, 2), all individuals of both species from Zhytomyr Region have metacentric chromosomes, whereas *V. contectus* from reservoirs of Lithuania and Italy have two pairs of submetacentric chromosomes and *V. viviparus* from Lithuania has only one such pair.

A visual analysis of the karyogram (fig. 1, 2) in both species shows no obvious pairs of chromosomes of the heteromorphic type ZW, and the type XY. This result confirms that the karyotype of these species, in contrast to some Western European representatives of the genus *Viviparus* (Baršienė et al., 2000), have no sex chromosomes. In this case, the findings are inconsistent with the data of O. V. Pavlyuchenkova (1997), pointing out the presence of a heteromorphic pair of XY-type in these mollusks.

A comparison of the measurements of homologous chromosomes in males and females of the two species shows significant differences between the sexes, confirmed by using the criteria of One-way ANOVA (table 1, 2). *V. viviparus* shows significant differences between three parameters of individual chromosomes: the absolute and relative length of chromosomes and the centromeric index, and only one, the relative length of chromosomes, in *V. contectus*. The relatively small number of significant differences in the latter species

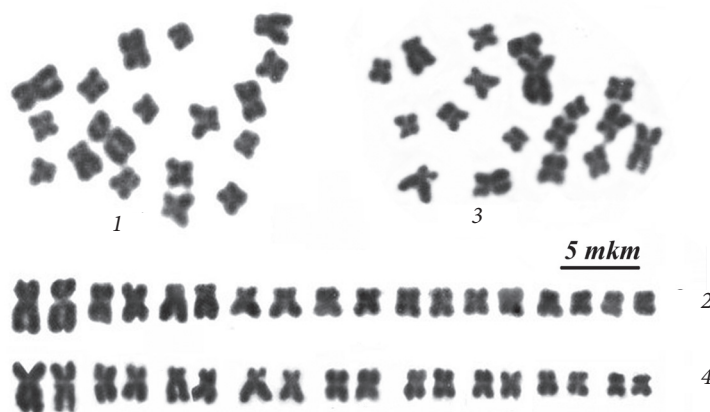


Fig. 1. Mitotic metaphases (1) and karyotypes (2) of female (above) and mitotic metaphases (3) and karyotypes (4) of male (below) of *V. viviparus*.

Рис. 1. Митотическая метафаза (1) и кариотип (2) самки (вверху); митотическая метафаза (3) и кариотип (4) самца (внизу) у *V. viviparus*.

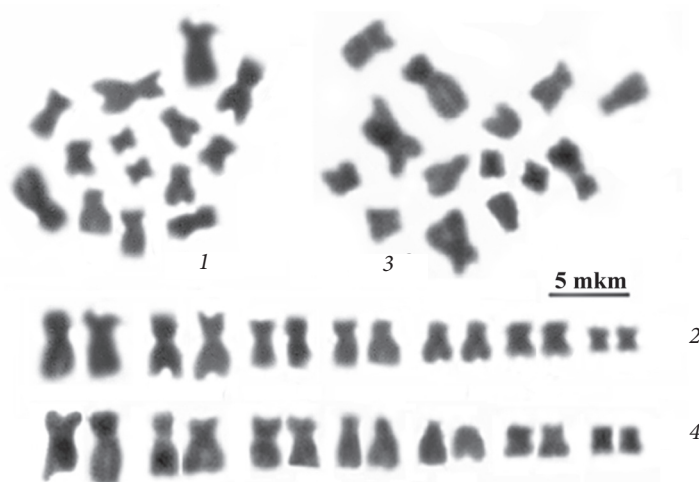


Fig. 2. Mitotic metaphases (1) and karyotypes (2) of female (above) and mitotic metaphases (3) and karyotypes (4) of male (below) of *V. contectus*.

Рис. 2. Митотическая метафаза (1) и кариотип (2) самки; митотическая метафаза (3) и кариотип (4) самца у *V. contectus*.

is apparently due to the smaller number of individuals in the sample compared to that of *V. viviparus*.

Discriminant analysis shows a high degree of isolation of chromosome sets of males and females in both species (table 3). Moreover, this pattern holds separately for each quantitative parameter, as well as for the three parameters as a whole. Levels of discrimination in all cases reach 100 % or are close to this figure. The discrimination of females and males in *V. contectus* is somewhat lower than that in *V. viviparus*.

We draw special attention to the character of the differences concerning the measurements and indexes of chromosomes: they involve both chromosomes of the same pair. Differences in the relative lengths of the chromosomes of males and females in *V. viviparus* are clearly evident when each of chromosomes of the 1, 2, 7, 8, and 9th pairs are considered (fig. 3); a similar trend in *V. contectus* holds for the pairs 4 and 6 (fig. 4).

The phenomenon of sex differentiation involving the length of autosomes is well known. In humans and rats (XY-sexing) the length of the longest pair of autosomes in males significantly exceeds that in females, while for the smallest pair of autosomes an

Table 1. Quantitative parameters of the karyotype in females and males of *V. viviparus*
Таблица 1. Количественные параметры кариотипа у самок и самцов *V. viviparus*

Chromosome pair	L				Ci, %				L, %			
	♀		♂		♀		♂		♀		♂	
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
1	5.00	0.16	5.45	0.23	38.03	0.95	36.89	1.05	17.69	0.46	19.40	0.35**
1	4.66	0.16	5.25	0.22*	38.72	0.62	35.80	1.36	16.47	0.42	18.67	0.33***
2	3.88	0.14	4.18	0.22	38.21	1.28	35.21	1.13	13.48	0.15	14.73	0.35**
2	3.75	0.14	3.98	0.20	37.61	1.45	32.40	1.59*	13.04	0.13	14.08	0.34**
3	3.60	0.13	3.65	0.17	38.35	1.47	36.66	1.11	12.63	0.11	12.92	0.31
3	3.47	0.14	3.54	0.16	37.27	1.12	34.55	1.35	12.29	0.20	12.56	0.30
4	3.30	0.14	3.20	0.16	38.67	0.94	38.09	1.02	11.50	0.13	11.34	0.26
4	3.11	0.15	3.15	0.18	40.78	0.89	38.45	1.69	10.98	0.24	11.19	0.50
5	2.89	0.16	2.89	0.14	42.99	0.93	38.97	1.28*	10.03	0.22	10.24	0.24
5	2.84	0.15	2.75	0.13	44.20	1.46	39.04	1.41*	9.88	0.12	9.75	0.20
6	2.76	0.14	2.65	0.13	41.93	0.92	39.11	1.27	9.59	0.11	9.39	0.22
6	2.62	0.14	2.54	0.13	42.21	1.31	38.01	1.52*	9.09	0.15	9.00	0.23
7	2.57	0.14	2.33	0.11	42.86	1.03	38.82	1.58*	8.91	0.17	8.31	0.24*
7	2.47	0.15	2.27	0.11	41.16	1.45	39.50	1.50	8.56	0.20	8.04	0.21
8	2.38	0.14	2.08	0.09	41.97	1.10	38.66	1.50	8.24	0.19	7.40	0.16**
8	2.28	0.14	1.97	0.08	41.00	1.21	37.31	1.39	7.86	0.20	7.07	0.19**
9	2.16	0.13	1.76	0.07*	39.82	1.34	40.48	1.44	7.50	0.19	6.34	0.20***
9	2.03	0.13	1.68	0.07**	42.44	1.37	40.33	1.15	7.00	0.20	5.98	0.14***

L — length of the chromosome; Ci, % — centromeric index; L, % — relative length of chromosomes; M — mean; SD — standard error of the mean.

Note. Statistical significance of differences between females and males: * — $p < 0,05$; ** — $p < 0,01$; *** — $p < 0,001$.

opposite pattern is observed (Evans, Swezy, 1928; Reitalu, 1968). Chromosomes of males are longer than those of females, what may be due to differences in the extent of their spiralization.

In humans, size differences can be observed between the chromosomes of one pair. In some cases, this is due to structural differences, in others can be caused by the varying degree of spiralization. For instance, the inactivated X chromosome of the female is shorter

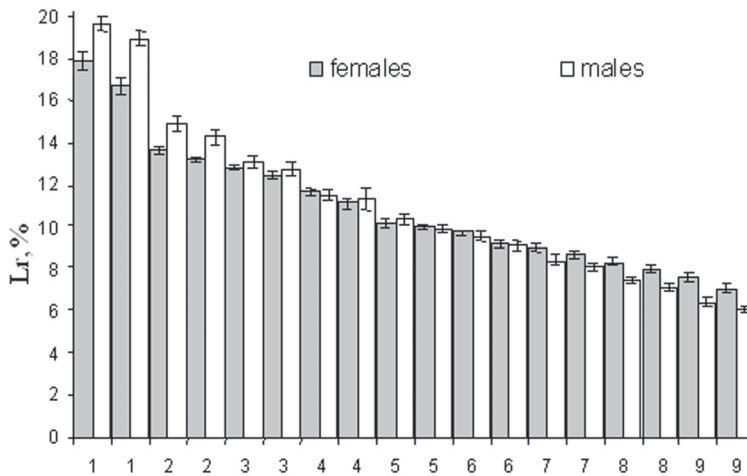


Fig. 3. Idiogramm of the relative chromosome length (L, %) in males and females of *V. viviparus*. X-axis — number of chromosome pair.

Рис. 3. Идиограмма относительной длины хромосом (L, %) у самок и самцов *V. viviparus*. По оси абсцисс — номер хромосомной пары.

Table 2. Quantitative parameters of the karyotype in females and males of *V. contectus*Таблица 2. Количественные параметры кариотипа у самок и самцов *V. contectus*

Chromosome pair	L				Ci, %				L, %			
	♀		♂		♀		♂		♀		♂	
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
1	6.06	0.76	5.30	0.42	39.67	1.61	34.95	2.09	23.62	0.96	21.83	0.52
1	5.82	0.69	5.17	0.42	38.06	1.72	35.62	1.41	22.83	0.94	21.29	0.55
2	5.04	0.72	4.71	0.40	35.64	2.67	37.98	2.44	19.13	0.44	19.30	0.32
2	4.87	0.71	4.62	0.40	36.82	2.38	37.05	2.02	18.49	0.43	18.92	0.37
3	4.24	0.63	3.83	0.32	39.54	1.43	35.10	2.65	15.97	0.62	15.77	0.54
3	4.08	0.61	3.64	0.30	36.33	1.93	35.87	2.13	15.42	0.69	15.01	0.58
4	3.12	0.46	3.22	0.26	37.53	2.35	32.28	2.51	12.04	0.54	13.26	0.25
4	3.10	0.42	3.14	0.27	36.22	2.72	33.54	2.25	11.90	0.32	12.87	0.24*
5	2.88	0.36	2.70	0.19	37.30	2.05	39.06	3.43	11.18	0.31	11.19	0.27
5	2.75	0.35	2.61	0.18	39.42	2.68	35.53	3.02	10.70	0.37	10.82	0.30
6	2.57	0.37	2.49	0.18	39.73	3.29	37.29	3.78	9.71	0.29	10.26	0.20
6	2.37	0.34	2.41	0.19	38.90	4.21	36.30	4.15	9.03	0.23	9.90	0.24*
7	2.19	0.32	2.07	0.16	36.69	2.60	40.81	3.04	8.33	0.32	8.51	0.31
7	2.08	0.28	1.98	0.16	40.51	2.22	41.70	2.44	8.04	0.50	8.17	0.34

L — length of the chromosome; Ci, % — centromeric index; L, % — relative length of chromosomes; M — mean; SD — standard error of the mean.

than the active X chromosome (Gilbert-Dreifus et al., 1963), individuals XXXXY have three inactivated X chromosomes of different length and are shorter than the active X chromosome (Atpins et al., 1963). X chromosome inactivation is a classical example of the transition between active and inactive status of chromatin (facultative heterochromatin). It is likely that the observed differences in the sizes of chromosomes in *V. contectus* and *V. viviparus* are also related to differences in the organization of facultative heterochromatin in males and females.

In accordance with modern ideas about the evolution of sex chromosomes (Graves, 2006), this process passes through several regular stages: the appearance of a sex-determining locus on one of the homologues; accumulation of sex-determining alleles; suppression of recombination and the emergence of chromosome-specific areas; degeneration of the specific area in Y(W) chromosomes with the exception of the pseudoautosomic portion; degeneration of the whole Y(W) chromosome. Since the genus *Viviparus* contains species for which sex is determined by heteromorphic chromosomes, it can be assumed

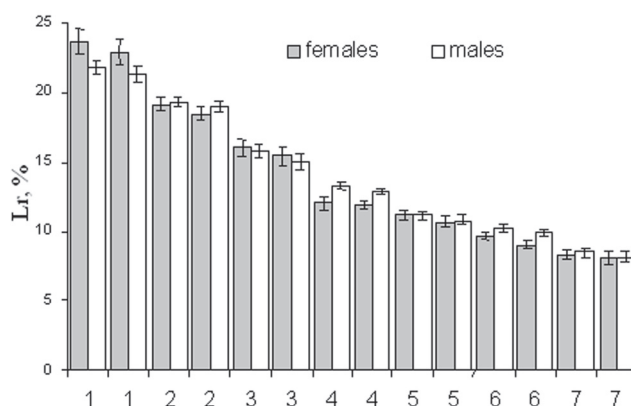


Fig. 4. Idiogram of the relative chromosome length (L, %) in males and females of *V. contectus*. X-axis — number of chromosome pair.

Рис. 4. Идиограмма относительной длины хромосом (L, %) у самок и самцов *V. contectus*. По оси абсцисс — номер хромосомной пары.

Table 3. Matrix of discrimination by different parameters of the karyotype and total

Таблица 3. Матрицы дискриминаций по разным параметрам кариотипа и в целом

Discrimination groups	<i>V. contectus</i>			<i>V. viviparus</i>		
	%	♀	♂	%	♀	♂
L						
$\frac{O_3+O}{O_3}$	100	9	0	95,2	20	1
$\frac{O_3+O}{O_3}$	100	0	9	85,7	3	18
Total	100	9	9	90,5	23	19
c, %						
$\frac{O_3+O}{O_3}$	89	8	1	85,7	18	3
$\frac{O_3+O}{O_3}$	100	0	9	71,4	6	15
Total	94	8	10	78,6	24	18
L, %						
$\frac{O_3+O}{O_3}$	100	9	0	95,5	21	1
$\frac{O_3+O}{O_3}$	100	0	9	90,5	2	19
Total	100	9	9	93,0	23	20
L + c, % + L, %						
$\frac{O_3+O}{O_3}$	100	0	9	100	21	0
$\frac{O_3+O}{O_3}$	100	9	0	100	0	21
Total	100	9	9	100	21	21

L — length of the chromosome; c, % — centromeric index; L, % — the relative length of chromosomes; L + c, % + L, % — total by three indices.

that species in which such chromosomes are not detected are close to this stage in the evolution of chromosomes. Thus, the determination of sex in *V. contectus* and *V. viviparus* is very likely provided by a homomorphic sex chromosome pair with sex-specific alleles. Underdeveloped mechanisms of sex determination involving both species also suggests a certain amount of environmental influence.

This fact shows that the differences in the quantitative features of male and female chromosomes of these species are associated not with the presence of heteromorphic pairs, but with changes involving the dimensions and proportions of chromosomes of a specific chromosome pair. Thus, differences in the karyotypes of the two sexes are caused not by the presence of sex chromosomes, but most likely by peculiarities of the functioning of the genetic apparatus of males and females. This means that for the two species of mollusks epigamous sex determination takes place in which the sex ratio in the population is representative of the habitat, and the revealed differences in the karyotypes of males and females are of physiological nature and akin to sexual dimorphism at the level of anatomical features. In addition the specific mechanism of these differences can be associated with a varying degree of spiralization of the chromosomes during mitosis or the packing of chromatin in males and females.

The obtained results show that the differences in the sex ratio in the populations of the two studied species of mollusks are associated not with the peculiarities of sex determination, but obviously with the degree of suitability of their environment. A dramatic shift in the sex ratio is observed both for *V. viviparus* and *V. contectus* in Ukraine (Andriychuk, Mezhzherin, 2013), indicating the poor condition of populations of these species in the waters of Ukraine (Mezhzherin et al., 2014), in contrast to other river systems, for instance such as in Poland (Jakubik, 2012), where the ratio of females to males is more close to 1 : 1. In this case, the environmental conditions prevailing in aquatic ecosystems of Ukraine are particularly unfavorable for *V. contectus*, populations of which are noticeable for their deficit of males.

Whereas adverse environmental conditions cause a shortage of males in the populations of the studied mollusks, populations of bony fish more often show an opposite trend. Numerous experimental studies (Devlin, Nagahama, 1996; Baroiller, D'Cotta, 2001; Devlin, Nagahama, 2002) and observations involving natural populations (Altukhov et al., 1989) show that environmental stress (a sharp rise in the temperature of incubation,

changes of the pH of the water, poor food base or high population density) tend to lead to an increase in the proportion of males. Under extreme conditions this may even result in offspring consisting of males alone. From this we can conclude that epigamous mechanisms of sex determination are very diverse and depend on the position of the organism in the evolutionary system.

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