

Research Article

Sex, size and ploidy ratios of *Carassius gibelio* from Poland

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Abstract

Unusual tolerance of ecological conditions and an ability of bisexual and unisexual reproduction allows *Carassius gibelio* (Bloch, 1782) to colonize various habitats from northern Europe to Asia. This non-native species of negligible economic importance has a negative impact on native species and the environment, and is undesirable in any part of Europe. As the reproductive mode is indirectly indicated by sex and ploidy ratios, these two features together with the karyotypes as well as sex- and ploidy-related length-weight relationships were studied in *C. gibelio* population inhabiting the Siemianówka Reservoir (northern-east Poland). Moreover, we tested the hypothesis that the frequency of triploid or diploid *C. gibelio* individuals in European populations will be greater than that in East Asia where it is native. The studied population is bisexual, clearly indicated by the presence of females (the sex ratio 3.1:1), represented by triploids and diploids in a 1:1 ratio. The parity of the sex ratio among diploids was noticeable in that most males (78.8%) were diploids but, surprisingly, the rest were triploids. The sex and ploidy ratios indicated both unisexual (via gynogenesis) and bisexual reproduction of *C. gibelio*. The weight-length relationships were related to sex but not to the ploidy. Compared to native populations, our findings reveal a higher incidence of sexually-reproducing diploids, which may have facilitated their success in invading Europe.

Key words: adaptability, Cyprinidae, karyotype, non-native species, polyploidy, unisexual gynogenesis, bisexual reproduction, weight-length relationship

Introduction

The gibel carp (Prussian carp, silver crucian carp) *Carassius gibelio* (Bloch, 1782) is a polyploid species of the family Cyprinidae. Definite data on its original distribution in Europe are not available (Rylková et al. 2013). However, it is usually considered to have been introduced to Europe with East Asian cyprinids (Takada et al. 2010) and probably appeared in open waters through accidental escape from aquaculture (Kalous et al. 2012). The economic importance of this species is negligible, but its adverse effects on native species, e.g. the crucian carp *Carassius carassius* (Linnaeus,

Table 1. The sex ratio and ploidy level of some native *C. gibelio* populations distributed in East Asia; F – females, M – males.

| (Number ^a) and name of localities | Sex | Ploidy level (number of localities ^a) | Sex ratio ^b F:M | Number ^b of males (%) | References |
|---|------|---|----------------------------|----------------------------------|--|
| North-eastern China | F | 3n | – | – | Shen et al. 1983; Shan and Jiang 1988, after Luo et al. 2014 |
| | M, F | 3n | – | – | |
| | M, F | 3n | – | – | Fan and Shen 1990 |
| Dongting water system, Hunan Province, China | M | 2n, 3n | 2n (1:1) | 11.9 | Xiao et al. 2011 |
| | F | 2n, 3n, 4n | 3n (5:1) | | |
| Changshan R., Zhejiang Province, China | M | 2n | 6.7:1 | 13.0 | Bai et al. 2011 |
| | F | 2n, 3n | | | |
| (4) ^a Xingkai Lake, Suifen River, Northeast Asia | M | 3n(3) | 3n (7:1) | 7.3–23.0 | Jiang et al. 2013 |
| | F | 3n(3) | | | |
| | M | 2n(1), 3n(1) | 2n (1:1.5) | 35.6 | |
| | F | 2n(1), 3n(1) | 3n (12.7:1) | | |
| (2) ^a Shuangfeng and Shuanglong Reservoirs, Heilongjiang Province, China | F, M | 3n | 4:1 | 20.0 | Luo et al. 2014 |
| (34) ^a Yellow, Yangtze and Pearl Rivers basins, northeast and northwest area of mainland China | M | 2n(7), 3n(22) | 82.3–2.8:1 | 1.2–26.5 | Liu et al. 2017a,b; Li et al. 2018 |
| | F | 2n(15), 3n(31) | | | |

a – number of localities if more than one

b – sex ratio and/or number of males has been given or calculated according to the published data.

1758), and the environment make it undesirable in any part of Europe (Hänfling et al. 2005; Wouters et al. 2012). Moreover, in aquaculture systems, *C. gibelio* is a competitor with cultures of the major reared species. In Poland, this species is considered a weed fish and a food competitor of the carp *Cyprinus carpio* Linnaeus, 1758 (Giosa et al. 2014).

Currently, *C. gibelio* is commonly regarded as a recurrent polyploidy hexaploid form of the *Carassius* species complex (Zhou and Gui 2017; Li et al. 2018), possessing a wide geographic distribution in the Eurasian continent and neighbouring islands (among others: Hänfling et al. 2005; Tóth et al. 2005; Liu et al. 2017a, b). Apart from hexaploids the *Carassius* species complex includes tetraploids and even octaploids (Gui and Zhou 2010; Takada et al. 2010; Jiang et al. 2013; Liu et al. 2017b) and *C. gibelio* forms of different ploidy are usually not discriminated morphologically from each other. The number of ca. 50 chromosomes is indicated as a diploid (2n) state of the fish (Leggatt and Iwama 2003), so tetraploids (4n) possess ca. 100 and hexaploids (6n) ca. 150 chromosomes. However, *Carassius* tetraploids have returned to a diploid state via rediploidization (2n = 100) and they reproduce bisexually. In turn, hexaploids, named also in comparison as triploids (3n = 150), reproduce by unisexual gynogenesis and bisexually, since males constitute a minor (up to 26.5%), but significant portion (Leonardos et al. 2008; Gui and Zhou 2010; Takada et al. 2010; Rylková et al. 2013; Jiang et al. 2013; Luo et al. 2014; Šimková et al. 2015; Zhang et al. 2015; Zhou and Gui 2017) (Table 1).

The advantages of dual mode of bisexual and unisexual reproduction in changing environmental conditions (Barbuti et al. 2012), which is rare among vertebrates, allow *C. gibelio* to adapt and colonize various habitats rapidly and achieve a wide range of distribution from northern Europe to

Asia (Gui and Zhou 2010; Jiang et al. 2013). It has also appeared recently in open waters of North America (Elgin et al. 2014). Such a successful invasion was also possible due to its unusual tolerance of a wide spectrum of ecological conditions (Vetemaa et al. 2005; Lusková et al. 2010; Grabowska and Przybylski 2015; CABI 2019). Among the suite of life-history traits which help fish to succeed in a new area, the style of reproduction is an important trait. Moreover, the sexually reproducing *C. gibelio* may create better adapted viable hybrids with *C. carassius* and other cyprinids (Wouters et al. 2012). Like other unisexual Teleostei, *C. gibelio* provides an opportunity to address two profound biological questions: the adaptive significance of sex and the mechanisms underlying the co-existence of bisexual and unisexual organisms that are closely related and live in close proximity (Schlupp 2005; Wootton and Smith 2014).

Until the 1990s, the majority of European *C. gibelio* populations were thought to be almost exclusively comprised by triploid females that arose through gynogenesis and represented discrete clonal lineages. The occurrence of males was subsequently reported in Hungary (Tóth et al. 2005), Estonia (Vetemaa et al. 2005; Tsoumani et al. 2006) and the Czech Republic (Lusková et al. 2010; Šimková et al. 2015). In Poland, only unisexual gynogenetic populations were initially reported, but later small numbers of males were discovered (Skóra 1971; Rokicki and Kulikowski 1994; Dąbrowski et al. 2012) and eventually entirely bisexual populations (Boroń 1994; Boroń et al. 2011) (Table 2). These reports imply that unisexual biotypes of *C. gibelio* make a temporal switch to partially (Liasko et al. 2010; Boroń et al. 2011) or predominantly sexually reproducing forms (Šimková et al. 2015). Such an ability to reproduce in different ways is not typical of other species, including native species, which reproduce only bisexually, or *C. gibelio* triploid females, which reproduce gynogenetically. The observed transition of *C. gibelio* European populations from unisexual to bisexual is apparent at the ploidy level, with triploid unisexual (gynogenetic) females ($3n = 150\text{--}160$) coexisting with sexual diploids ($2n = 100$) of both sexes and occasionally with a different number of triploid males (Tóth et al. 2005; Boroń et al. 2011; Jiang et al. 2013). Such mixed, diploid-polyploid populations composed of diploid and triploid forms are not so frequent in the home range of *C. gibelio*, e.g. in northeast Asia (Jiang et al. 2013; Liu et al. 2017a), and may indicate its invasiveness.

In different water bodies, environmental factors can affect fish in several ways, but fish responses can often be manifested in the size of individuals, i.e. maximum weight or length. As both traits are correlated, they are usually analysed as a weight-length relationship, which is an important component of fish life history and can potentially provide information about the functioning of a fish population (Froese 2006). Moreover, it was demonstrated that body size influenced the occurrence and colonization processes of alien species since larger taxa are less frequently subject to

Table 2. The sex ratio and ploidy level of some non-native *C. gibelio* populations distributed in Europe; F – females, M – males.

| (Number ^a) and name of localities | Sex | Ploidy level (number ^a) | Sex ratio ^c F:M | Number of males (%) | References |
|--|--------|-------------------------------------|----------------------------|---------------------|-----------------------|
| (7) ^a Freshwater lakes, ponds ^a , Estonia | F | – | 100 | 0.0 | Vetemaa et al. 2005 |
| (4) ^a Häädemeeste, Kasmu, Matsalu ^b , Saunja, Estonia | M F | – | 1.2–1.5:1 | 40.0–45.8 | |
| (9) ^a Greek lakes, Greece | F | – | 100 | 0.0 | Tsoumani et al. 2006 |
| | M | – | 38.0:1 | 2.6 | |
| Lake Pamvotis, Kalamas R., the Ionian Sea basin, Greece | M | 3n, 4n | 43.7:1 | 2.3 | Liasko et al. 2010 |
| | F | 3n | | | |
| Lake Chimaditis, Axios R., the Aegean Sea basin, Greece | F | – | 100 | 0.0 | Leonardos et al. 2008 |
| | M | 2n | 3.4:1 | 22.7 | Papousek et al. 2008 |
| F | 2n, 3n | | | | |
| Dyje River (lower part), adjacent floodplain, the Morava R., Black Sea basin, Czech Republic | M | 2n, 3n, 4n | 3.8:1 | 20.7 | Lusková et al. 2010 |
| | F | 2n, 3n, 4n | | | |
| Dyje River, the Morava R., Black Sea basin, Czech Republic | M | 2n | 3.2:1 | 23.6 | Šimková et al. 2015 |
| | F | 2n, 3n | | | |
| (12) ^a Eastern Ukraine | M | 2n(12) | – | – | Liasko et al. 2011 |
| | F | 2n(12), 3n(6) | | | |
| (14) ^a Croatia | M | 2n(11), 3n(4) | 3.3:1 | 21.6 | Jakovlić and Gui 2011 |
| | F | 2n(12), 3n(13) | | | |
| Mlynskie Lake ^b , Vistula R., Poland | M | – | 95:1 | 1.0 | Skóra 1971 |
| | F | – | | | |
| Zegrzynski Reservoir ^b , Vistula R., Poland | M | 2n | 4.8:1 | 18.8 | Boroń 1994 |
| | F | 2n, 3n | | | |
| (2) ^a Kisajno, Talty Lakes ^b , Vistula R., Poland | F | 3n | 100 | 0.0 | Boroń et al. 2011 |
| Goplo Lake, Warta R. ^b , Poland | M | – | 9.7:1 | 9.3 | Dąbrowski et al. 2012 |
| | F | – | | | |
| (3) ^a Leszczynskie Lakeland ^b , Vistula R., Poland | F | – | 100 | 0.0 | Giosa et al. 2014 |
| Siemianowka Reservoir ^b , Narew R., Poland | M | 2n, 3n | 3.1:1 | 24.5 | Present study |
| | F | 2n, 3n | | | |

a – number of localities if more than one.

b – Baltic Sea basin.

c – sex ratio has been given or calculated according to the published data.

predation (Leuven et al. 2017). This body parameter also affects competition with the native communities. Most published data refer to the size, i.e. body length (more frequent) and weight, or the relationship between these traits for *C. gibelio* populations of a known sex (e.g. Vetemaa et al. 2005; Tsoumani et al. 2006; Giosa et al. 2014) but rarely when both sex and ploidy are known (Liasko et al. 2010; Jakovlić and Gui 2011) (Table 2). Meanwhile, the mode of reproduction of *C. gibelio* expressed by sex and ploidy level seems to be one of the mechanisms underlying its invasion success (Leuven et al. 2017).

Preliminary results based on seven *C. gibelio* individuals from the Siemianówka Reservoir (Boroń et al. 2011) inspired the present study in which we report new data concerning sex and ploidy ratios of this first discovered and documented Polish *C. gibelio* population. A significant number of both females and males of different ploidy makes this population unique among others in Europe (Table 2) and has enabled us to conduct effective comparative studies. So, this paper contains the original and representative data of DNA content and karyotype patterns, including

the microchromosomes as well as rarely reported data of length and weight of *C. gibelio* for diploid and triploid individuals of both sexes. We tested whether sex or ploidy level is related to the length-weight relationships. The presence of triploid males seems to be particularly intriguing in the light of the sex determination mechanism in *C. gibelio* triploids and their reproductive mode transition (Zhang et al. 2015; Li et al. 2016, 2018) as well as in the context of the newly discovered "...a potential new mechanism in the evolution of sex determination system in polyploid vertebrates with unisexual gynogenesis ability..." (Li et al. 2018). We tested if the frequency of triploid or diploid females and males in European populations will be greater than previously reported in the home range of *C. gibelio* in East Asia. In this way, we indicated a new trait of this invading population, which seems to be a good indicator of their invasiveness. Thus, the presented results seem to contribute to our knowledge on invasion success of wide-spread non-native species.

Materials and methods

Ethic statement

Standard procedures used in aquaculture for catching fish were employed in the course of the study. Fish sampling and valid protocols for animal use in experiments were performed upon consent obtained from the Local Ethics Commission of the UWM in Olsztyn, Poland (no. 20/2013/N). The fin clips were taken from the dead fish. For chromosome slide preparations fish were sacrificed using an overdose of anaesthetics and kidney samples were taken from the dead fish.

Fish specimens and sex ratio examination

Samples of *C. gibelio* were collected from the Siemianowka Reservoir (Res.) (52°55'43.2"N; 23°48'38.2"E) at the Narew River in the Vistula River drainage (Baltic Sea basin) by commercial fishermen using monofilament gill nets with 50–70 mm mesh sizes or fyke nets. Altogether 136 *C. gibelio* individuals were collected in April, June and October in 2014 and 2016. These fish samples were selected with the attempts to collect a similar number of females and males for ploidy identification within sex. Fish were measured for standard (SL) and total (TL) lengths to the nearest 1 mm and were weighed (total weight, W) to the nearest 100 mg, and fin clips were collected. Solely for sex ratio analysis 200 individuals were randomly sampled in April 2016 and sex was indicated in living fish on the basis of evident external features resulting from sexual dimorphism (small, clearly visible white spawning rash on the heads and gill covers of males) and by a visual examination of the milt or released eggs. Apart from these features, *post mortem* gonad examination was used in the case of 136 individuals collected in 2014 and 2016.

Ploidy ratio among females and males, and size of *C. gibelio* were evaluated by combining individuals collected in 2014 and 2016. We did not anticipate marked differences within the samples collected in successive years. However, taking into account the fact that weight and length are related to comparison of differences within the samples collected in successive years, the analysis of covariance was used. Thus, the linear regressions of body weight (W) and the standard length (SL) for log-transformed data (Log) of females were $\text{Log } W = 2.192 \times \text{Log } SL - 1.825$ and $\text{log } W = 2.257 \times \text{Log } SL - 2.214$, respectively in 2014 and 2016 year, and the coefficients *a* (intercept) and *b* (slope) values did not differ ($t_{80} = 0.557$, $p = 0.340$ and $t_{78} = 0.955$, $p = 0.2513$, respectively). Similar results were noted for males, i.e. $\text{Log } W = 2.280 \times \text{Log } SL - 2.950$ in 2014 and $\text{log } W = 2.261 \times \text{Log } SL - 3.214$ in 2016 ($t_{47} = 0.4021$, $p = 0.365$ and $t_{45} = 1.480$, $p = 0.133$ for comparisons *a* and *b* respectively).

In addition to *C. gibelio*, the fish assemblage in the studied reservoir contains predominantly native species, such as: pikeperch *Sander lucioperca* (Linnaeus, 1758), pike *Esox lucius* Linnaeus, 1758, European perch *Perca fluviatilis* Linnaeus, 1758, ide *Leuciscus idus*, (Linnaeus, 1758), tench *Tinca tinca* (Linnaeus, 1758), roach *Rutilus rutilus* (Linnaeus, 1758), ruffe *Gymnocephalus cernuus* (Linnaeus, 1758), freshwater bream *Abramis brama* (Linnaeus, 1758), crucian carp *C. carassius*, European eel *Anguilla anguilla* (Linnaeus, 1758) and European catfish *Silurus glanis* Linnaeus, 1758, and non-native, viz. common carp *C. carpio*.

To distinguish *C. gibelio* from a relative native species *C. carassius*, species-specific features of all individuals were verified according to external morphology (silvery-brown colour of the body, strongly serrated last unbranched ray in the anal and dorsal fin, concave or straight the external margin of the dorsal fin, the number of 37–52 gill rakers, lateral line with 29–33 scales; anal fin with 5½ branched rays) and dark colour of peritoneum (Figure 1) (Kottelat and Freyhof 2007; Boroń et al. 2011). The affiliation of the investigated *C. gibelio* individuals to the same species is also indicated by an analysis of the 5S rRNA nucleotide composition, as described by Szabelska et al. (2017).

Sex ratio was calculated as the number of males divided by the total number of individuals (males plus females) in the population. For each site date set, the sex ratio was tested for deviations from a mendelian parity (1:1 sex ratio) using a binomial test (Wilson and Hardy 2002). The same test was used to compare the occurrence frequency of diploids and triploids among *C. gibelio* native populations in East Asia and in non-native populations in Europe, taking into account the present and formerly published data shown in Tables 1 and 2.

Ploidy determination

The ploidy level of all 136 individuals was determined by chromosome count (95 individuals) and measurement of nuclear DNA content of fin cells

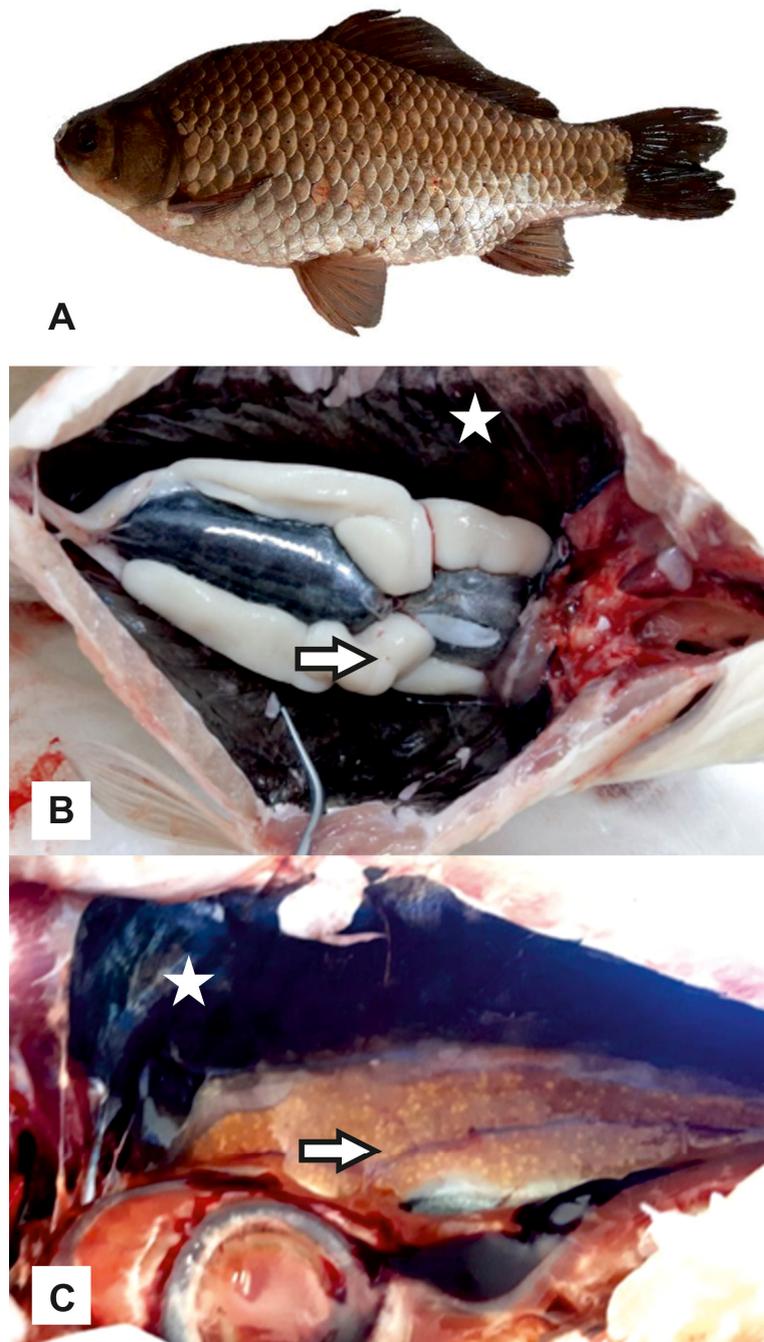


Figure 1. Gibel carp *C. gibelio* from the Siemianówka Res. (A), the interior of the body of male (B) and female (C); asterisk indicates the dark-coloured peritoneum, arrowhead indicates testis and ovary, respectively. Photo by A. Przybył.

using flow cytometry (59 individuals). Some of the triploids (18 individuals) were verified using both of these techniques. Chromosome slides were prepared according to Boroń et al. (2011). Live fish were first injected with a dose of 1 ml of 0.05% colchicine solution per 100g body weight. After 1.5 hours the fish were anaesthetized using MS222 prior to sacrificing. Mitotic chromosomes were obtained from kidney cells that were exposed to a hypotonic solution (0.075M KCl) for 30 min and fixed in methanol : acetic acid (3:1). Chromosomes were stained with a solution of 4% Giemsa (pH = 6.8). At least 10 metaphase spreads from each individual were studied

using a Nikon Eclipse 90i fluorescence microscope equipped with ProgResMFcool camera (Jenoptic). The images were processed using MultiScan software with the Karyotype supplement. Metaphase chromosomes were classified using the nomenclature proposed by Levan et al. (1964) into the morphological categories: metacentrics (m), submetacentrics (sm), subtelo- and acrocentrics (sta). In order to provide the number of chromosome arms, it was assumed that the first two categories (m, sm) belong to bi-armed chromosomes, while the other (sta) belongs to uni-armed chromosomes.

Fin clips were fixed in 100% ethanol and placed in 70% ethanol at -80°C . Flow-cytometry used the Becton Dickinson FACScalibur process. Fin tissue was chopped in small pieces in phosphate-buffered saline (PBS; Biomed Lublin, Poland), filtered and stained in 1 ml of nuclear staining solution (NSS; 10 mg propidium iodide, 20 μl 1.12% trisodium citrate dihydrate, 60 μl Nonidet P40 (Sigma) per 200 ml distilled water). The samples were centrifuged for 5 min at 700g at room temperature (RT) and the cell pellets were re-suspended in the 250 μl NSS and incubated for 15 min (RT in dark). For each sample of 250 μl working solution, ribonuclease A from bovine pancreas (Sigma) was added and incubated for the 15 min (RT in darkness). The erythrocytes of chicken (*Gallus gallus domesticus*) were used as the internal standard. Single cell suspensions were obtained by applying the same method as for fin clips. For measurement, fish cells and chicken erythrocytes were mixed to a similar final concentration. For each sample, information on at least 10,000 nuclear events was acquired using the CELLQuest software program (Becton Dickinson Bioscience). For data analysis, the ModFitLT V3 (PMac) software program was used. The DNA content of the standard was considered as 2.5 pg of DNA per nucleus (Vinogradov 1998). The difference in nuclear DNA content of males and females of different ploidy was tested using ANOVA I, following the Fisher least significant differences (thereafter LSD) post-hoc test.

Weight-length relationship

The relationship between SL and TL was estimated by reduced major axis, i.e. regression (type II); without specification of independent and response variable (Smith 2009). Differences in SL between sex and ploidy level were examined using hierarchical (nested) analysis of variance where ploidy level was nested in sex. Since fish weight varies strongly between seasons, mainly due to gonad development (Wootton and Smith 2014), the weight-length relationship was calculated only for samples taken in October, i.e. after the spawning season. The relationship between SL and W of fish was assessed through linear regressions of log-transformed data, $\text{Log } W = \text{Log } a + b \text{ Log } \text{SL}$, where the coefficient a (intercept) describes the rate of change of weight with length and the coefficient b (slope) provides information on the isometric or allometric growth pattern (Le Cren 1951).

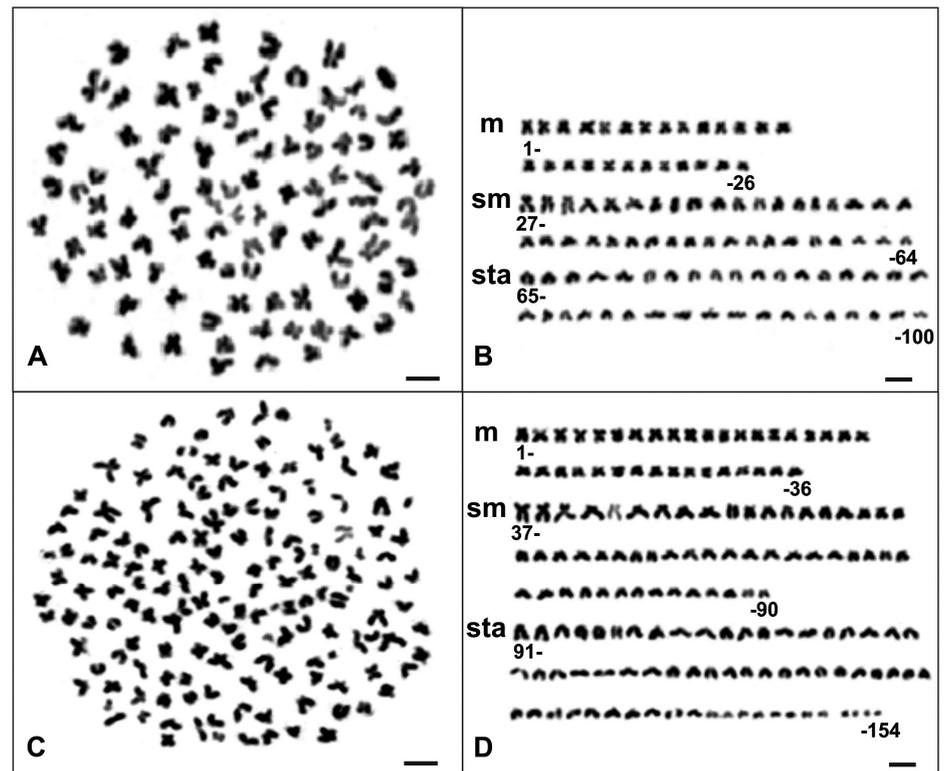


Figure 2. Representative mitotic metaphase stained with Giemsa and corresponding karyotype of *C. gibelio* diploid $2n = 100$ (A, B) and triploid $3n = 154$ (C, D) female. Symbols of chromosomes: m – metacentric, sm – submetacentric, sta – subtelo-acrocentric. Bar equals $10 \mu\text{m}$.

To examine whether the weight-length relationship was isometric ($b = 3$) or allometric (negative, $b < 3$ or positive allometric, $b > 3$), a t-test was used. An analysis of covariance (ANCOVA), following the multiple comparisons procedure, was used to test differences in the weight – length (SL) linear regressions in response to sex and ploidy. When the null hypothesis was rejected, the post-hoc Tukey honestly significant differences (thereafter HSD) post-hoc test was used to identify a regression responsible for the observed differences. Before running all ANOVA, the data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene’s test). Statistical analyses were performed in Statistica 12 for Windows, StatSoft Inc.

Results

Ploidy identification and karyotype structure

Among the 136 individuals of *C. gibelio*, 84 (61.8%) were diploid ($2n$) and 52 (38.2%) were triploid ($3n$). A diploid chromosome number of 100 elements was found in 94.1% out of 656 analysed metaphase plates of 64 individuals. In the remaining metaphases, 97–99 chromosomes were observed. The karyotype structure of both diploid ($2n = 100$) males and females was the same and included 26 m, 38 sm and 36 sta chromosomes and the chromosome arm number (NF) was 164 (Figure 2A, B).

Table 3. Nuclear DNA content of the cells of diploid (2n) and triploid (3n) *C. gibelio* males (M) and females (F); N – number of individuals, SD – standard deviation. a, b – the same letters denote groups that do not differ statistically (LSD Fisher post-hoc test).

| Ploidy and sex | N | DNA content (pg) | |
|----------------|----|------------------|---------------------------------|
| | | Range | Mean (\pm SD) |
| 2n M | 12 | 4.67–5.25 | 4.83 (\pm 0.17) ^a |
| 3n M | 9 | 6.55–7.75 | 7.23 (\pm 0.40) ^b |
| 2n F | 8 | 4.55–5.20 | 4.73 (\pm 0.21) ^a |
| 3n F | 30 | 6.40–7.52 | 7.10 (\pm 0.25) ^b |

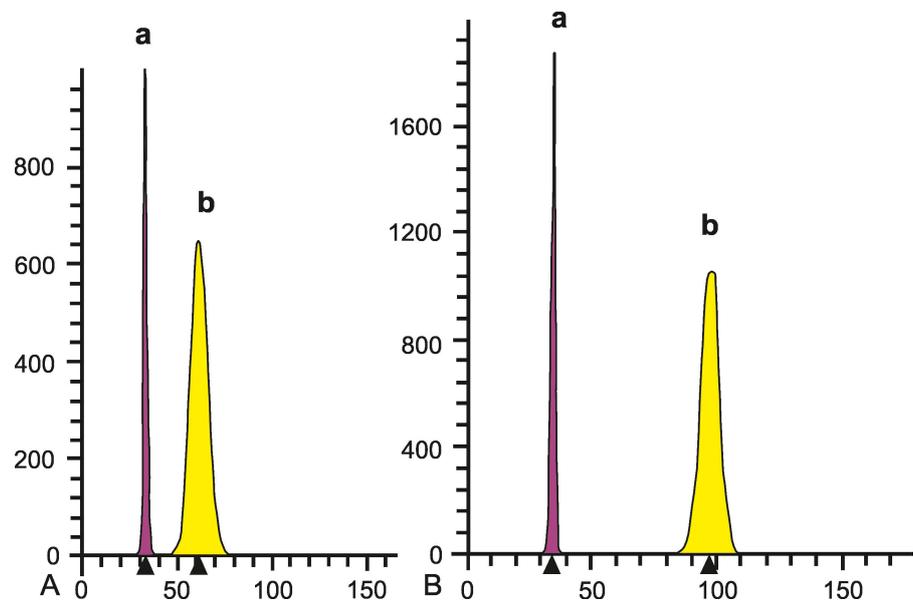


Figure 3. Flow cytometric histograms for relative DNA content of fin cells of diploid (A) and triploid (B) *C. gibelio*. Y-axis and X-axis show cells and channel number, respectively. Peak (a) shows the DNA content of chicken erythrocytes (2.5 pg) used as a reference standard control. Peak (b) refers to cells of 2n and 3n *C. gibelio*, respectively.

A triploid number from 150 to 164 chromosomes in 95.4% out of 323 analysed metaphase plates of 31 individuals was observed. In the remaining metaphases from 135 to 139 chromosomes were counted. The karyotype of 3n females was composed of a modal number of 3n = 154 chromosomes; 36 m, 54 sm and 64 sta, NF of 244 (Figure 2C, D). However, the karyotype of 11 triploid males contained the modal number of 3n = 160 chromosomes, categorized into 34 m, 58 sm and 68 sta, NF of 246 (not show here). From zero to six, but most frequently, four microchromosomes were detected (Figure 2) that could not be classified in any morphological category in the karyotypes of 3n females and males.

Among 59 individuals analysed by measuring nuclear DNA contents, 20 diploids and 39 triploids were detected (Table 3). Their representative histograms are displayed in Figure 3. Mean (\pm SD) cell DNA contents of 2n was 4.79 pg (\pm 0.190), while for 3n specimens it was 7.13 pg (\pm 0.295). The DNA content of fish of different ploidy and sex differed significantly ($F_{3,55} = 345.53$, $p < 0.001$) but multiple comparisons (LSD Fisher test) revealed that 3n females and males had higher DNA concentrations than 2n ones (3n F = 3n M > 2n F = 2n M). Mean (\pm SD) cell DNA contents of 3n were about 1.5 times higher than that of diploids.

Table 4. The standard length (SL) and weight (W) of *C. gibelio* (N – number of individuals) in the Siemianówka Res. (average, SD – standard deviation and range) according to ploidy level (2n – diploid; 3n – triploid) and sex (F – female; M – male).

| ploidy and sex | N | SL (mm) | | | | W (g) | | | |
|----------------|-----|---------|------|-------|-------|---------|-------|-------|--------|
| | | average | SD | min | max | average | SD | min | max |
| 2n F | 43 | 272.8 | 43.4 | 200.0 | 400.0 | 769.8 | 405.3 | 290.1 | 2035.0 |
| 2n M | 41 | 248.1 | 32.6 | 160.0 | 330.0 | 520.6 | 169.4 | 135.3 | 962.4 |
| 3n F | 41 | 265.3 | 33.2 | 193.0 | 330.0 | 664.8 | 201.6 | 276.4 | 1196.5 |
| 3n M | 11 | 240.3 | 25.4 | 197.0 | 275.0 | 517.8 | 138.7 | 296.7 | 677.1 |
| total | 136 | 260.5 | 37.6 | 160.0 | 400.0 | 642.6 | 290.1 | 135.3 | 2035.0 |

Sex and ploidy ratios

Among 200 individuals collected for sex examination 151 were recognized as females and 49 as males (24.5%). Thus, the sex ratio ($f_M = 0.245$ or 3.1:1) was significantly biased towards females ($p < 0.001$). In total, 61.8% of *C. gibelio* individuals were 2n (Table 3) and taking into account only diploid fish the sex ratio did not differ from the parity ($f_M = 0.485$, $p > 0.05$). The ploidy structure among females did not deviate from 1:1 ($p > 0.05$). In contrast, 21.2% of males were 3n and the 2n:3n ratio among males was 3.7:1, which was significantly different from parity ($p < 0.001$). Triploids were significantly more frequent among females than males ($p < 0.001$).

Among 45 populations examined in East Asia (Table 1) triploid males were much more frequent ($f_{3nM} = 0.689$) than diploid males ($f_{2nM} = 0.244$) ($p < 0.0001$) and a similar pattern was noted for females, i.e. $f_{3nF} = 0.933$ and $f_{2nF} = 0.400$, respectively ($p < 0.0001$). On the contrary, in 34 examined European locations (Table 2), diploid males were noted in 28 locations ($f_{2nM} = 0.824$), but triploid males were found in only 3 populations ($f_{3nM} = 0.206$). Meanwhile, the frequency of the occurrence of triploid and diploid females was the same ($f_{2nF} = 0.853$, $f_{3nF} = 0.794$; $p = 0.5248$). Comparisons of the frequency of the occurrence of males and females of different ploidy in two areas of *C. gibelio* distribution revealed that diploid males and diploid females were noted more frequently in Europe ($p < 0.0001$ and $p < 0.0001$, respectively) whereas triploid males were noted more frequently in East Asia ($p < 0.0001$). However, there were no significant differences in the occurrence of triploid females in both areas ($p = 0.065$).

Length and weight relationships

The relationship between standard (SL) and total (TL) length (major reduced axis) was $TL = 8.645 + 1.178 \times SL$ ($r = 0.9213$, $n = 84$, $p < 0.001$) for females and $TL = 8.6848 + 1.2513 \times SL$ ($r = 0.9692$, $n = 52$, $p < 0.001$) for males. The data of SL and W of *C. gibelio* females and males of different ploidy are given in Table 4. Nested analysis of variance revealed that differences in SL were related to sex ($F_{1,132} = 11.651$, $p < 0.001$) but not to the ploidy level ($F_{2,132} = 0.658$, $p = 0.5196$) and LSD post-hoc test showed that females are larger than males ($2nF = 3nF > 2nM = 3nM$).

Table 5. Regression parameters and their standard errors (s.e.) of body weight on standard length (log-transformed data) for *C. gibelio* from the Siemianowka Res. for diploid (2n) and triploid (3n) females (F) and males (M).

| ploidy and sex | intercept a | s.e. a | slope b | s.e. b | coefficient of determination r^2 | sample size N |
|----------------|-------------|--------|---------|--------|------------------------------------|---------------|
| 2n F | -5.3159 | 0.4950 | 3.3709 | 0.2071 | 0.9331 | 21 |
| 3n F | -3.5123 | 0.8519 | 2.6262 | 0.3587 | 0.7702 | 18 |
| 2n M | -4.9484 | 0.3326 | 3.2106 | 0.1410 | 0.7702 | 12 |
| 3n M | -3.5057 | 1.2892 | 2.6119 | 0.5453 | 0.7927 | 8 |

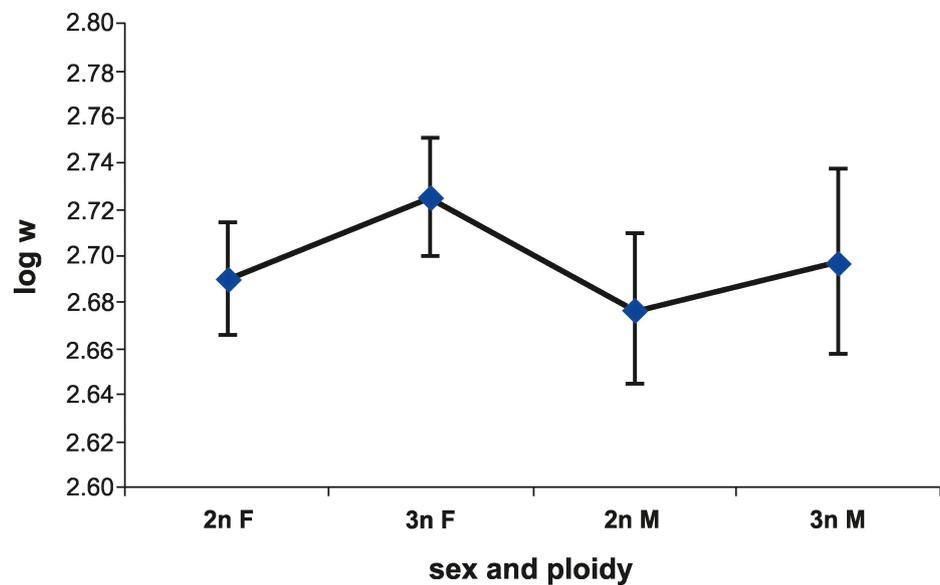


Figure 4. The average weight and its 95% C.L. (log-transformed data) of different sex and ploidy *C. gibelio* calculated for 237 mm average SL.

There were strong relationships between SL and W for all individuals of both sexes (Table 5). The covariance analysis revealed a lack of difference in the slopes of this relationship ($F_{3,41} = 1.779$, $p > 0.1$) and the common slope for all individuals was $b_c = 3.10$ (s.e. $b_c = 0.146$). The common slope did not differ significantly from 3 ($t = 0.688$, $df = 41$, $p > 0.05$), which means that the weight-length relationship was isometric. However, these regressions differed in their intercept coefficients ($F_{3,43} = 3.255$, $p < 0.05$) and multiple comparisons (HSD Tukey test) showed a lack of differences in intercept between ploidy levels, but differences were observed between the sexes ($2nF = 3nF > 2nM = 3nM$) (Table 5). Thus, taking into account the mean SL = 237 mm for all compared groups related to sex and ploidy, females (2n, 3n) were significantly heavier than males (2n, 3n) (Figure 4).

Discussion

There is no historical information for *C. gibelio* introduction to the Siemianówka Reservoir. Most probably it appeared in small water bodies, such as an oxbow lake or pond located in the area flooded with water, or entered during restocking with carp or was introduced by anglers. *C. gibelio* has no breeding significance in Europe, but some of its gynogenetic clones characterized by greater size and resistance to disease are grown in Asia (Mei and Gui 2015; Li and Gui 2018).

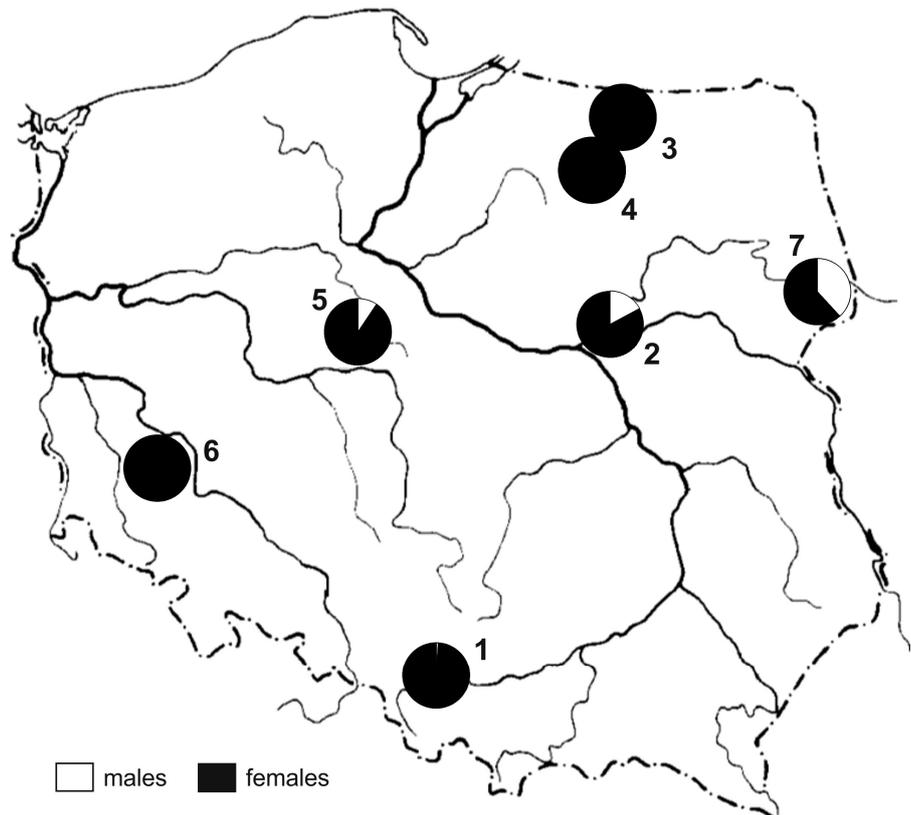


Figure 5. Sex ratio of some Polish *C. gibelio* populations; females and males are shown respectively as the black and white areas of pie charts, numbers represent data source: 1 – Mlynskie Lake (Skóra 1971), 2 – Zegrzyński Res. (Boroń 1994), 3 – Kisajno Lake and 4 – Talty Lake (Boroń et al. 2011), 5 – Gopło Lake (Dąbrowski et al. 2012), 6 – Leszczyńskie Lakeland (Giosa et al. 2014), 7 – Siemianówka Res. (present study).

Sex and ploidy structure of C. gibelio populations

In almost all surveys of mixed populations of *C. gibelio* in Europe there are 2n and 3n females and 2n males (Table 2). Data published so far, supported by the presented results, indicate an increasing number of males and suggest a transition of *C. gibelio* populations from unisexual gynogenetic to sexual modes of reproduction. The latter mode exists in a range of environments; in salt water (Vetemaa et al. 2005), in rivers (Lusková et al. 2010; Šimková et al. 2015) and in lakes and reservoirs (Boroń 1994; Boroń et al. 2011; present study). Some of the European bisexual *C. gibelio* populations display more than 10% of males characterized by females to male ratio ranging from 11.4:1 (Lusková et al. 2010) to 1.2:1 (Vetemaa et al. 2005) (Table 2). With regard to the participation of males, several of these populations (Papousek et al. 2008; Lusková et al. 2010; Jakovlić and Gui 2011; Šimková et al. 2015) are similar to the two Polish ones discovered so far in the Zegrzyński and Siemianówka Reservoirs (Boroń 1994; Boroń et al. 2011; present study) (Table 2, Figure 5). The sex and ploidy ratio in *C. gibelio* populations distributed in Poland appears dynamic and it seems to be unrelated to the geographical distribution from the south to the north proposed by Giosa et al. (2014).

Changes in ploidy ratios may have facilitated the invasion of *C. gibelio*. Demonstrated in this paper, the incidence of diploid males (M 2n) and females (F 2n) in Europe is significantly higher than in East Asia. Interestingly, this process is evident in Ukraine and South Russia, where diploid populations gradually displaced triploids and become dominant in almost all freshwater ecosystems (among others, Liasko et al. 2011). As this does not happen within the native range, it is relevant to invasion.

In turn, triploid *C. gibelio* females are still dispersed widely in Europe and they are not less frequent here as in East Asia, deriving ecological benefits from a significantly larger genetic variation and stronger environmental adaptation in comparison with diploids (Jiang et al. 2013; Zhou and Gui 2017; Liu et al. 2017a). Interestingly, in *Carassius* species complex from 34 localities in China, in total triploids (6n) were 2.5 times more than diploids (4n) and they had wider geographic distribution, whereas only nine localities were dominated by diploids (Liu et al. 2017a). Triploid males were frequent in 30 out of 45 populations/localities containing 3n females, distributed in East Asia analysed under the present study (Table 1). Moreover, recent reports indicated multiple independent polyploidy origins of triploids (= hexaploids) from sympatric diploids (tetraploids) in *Carassius* species complex across East Asia (Liu et al. 2017b; Li et al. 2018). Furthermore, 3n *C. gibelio* distributed in China “might be under the evolutionary trajectory of the reproduction mode transition from unisexual to bisexual reproduction” (Zhang et al. 2015; Li et al. 2016, 2018). So, triploids now seem to benefit from being polyploid by reaching stable bisexual populations with two alternative modes of reproduction. In the same time, in the European range of *C. gibelio* distribution, diploids seem to benefit from bisexual reproduction, whereas coexisting 3n females still reproduce gynogenetically that 3n males are rare (Table 2).

The indicated heterogeneity in ploidy levels among males in the Siemianówka Res. confirmed our previous findings (Boroń et al. 2011) and is consistent with data on European populations located more to the south (Tóth et al. 2005; Liasko et al. 2010; Lusková et al. 2010; Jakovlić and Gui 2011). The observed diversity of ploidy in these populations seems to be correlated with the hypothesis that this species is required to exert more reproductive effort to invade natural ecosystems than artificial waters (Tarkan et al. 2012). However, only a few males occurring in populations dominated by females have gonads showing normal development (Vetemaa et al. 2005; Liasko et al. 2010; Dąbrowski et al. 2012). On the contrary, triploid males in the Siemianówka Res. participated in spawning that their gonads were properly developed and all of them regardless of ploidy were observed to release milt during the spawning period (Boroń et al. 2011; present study).

The occurrence of ploidy diversity among males in the gonochoristic – gynogenetic populations suggests the involvement of multiple reproductive

mechanisms. Triploid males could result from environment-dependent sex determination (Devlin and Nagahama 2002; Li et al. 2018; Li and Gui 2018). Moreover, sex determination genes of males might still be present in the genome of females (Jiang et al. 2013; Mei and Gui 2015) and might be activated by some environmental factors, such as temperature changes as the proportion of temperature-dependent sex determination in *C. gibelio* hexaploids was positively correlated to annual temperature in wild populations (Li et al. 2018). It was also suggested that some eggs of *C. gibelio* are capable of passing through meiosis whereas triploid males produced aneuploid ($1.5n$) spermatozoa (Zhang et al. 2015). Triploid males seem to play an important role in increasing the size and genetic variation of populations in East Asia (Jiang et al. 2013; Zhou and Gui 2017; Li et al. 2018). In addition, recently published data show that “the extra microchromosomes” with repetitive sequences and transposable elements in males might resemble a common feature of sex chromosomes and play male determination role in the bisexually reproduced progenies during the evolutionary trajectory of the reproduction mode transition from unisexual gynogenesis to bisexual reproduction in polyploid fish (Li et al. 2016, 2018; Li and Gui 2018). Further monitoring of the sex and ploidy ratios in *C. gibelio* populations following a gynogenetic-sexual transition is required, particularly for populations currently containing males (Table 2). The population under present study seems to be a good model also in the context of the potential effects of global warming to temperature-dependent sex determination in *C. gibelio* triploids. Furthermore, the observed appearance of triploid males in the European populations (Table 2) may be a response of this species like other predicted responses of non-native species to warming temperatures in a temperate region (Britton et al. 2010).

Triploids of *C. gibelio*, unlike other vertebrates, possess dual modes of unisexual gynogenesis and bisexual reproduction “that efficiently help them form a bypass to the fatal end of evolution and endows it with evolutionary potentials by introducing fresh recombined genetic material into the frozen ones” (Jiang et al. 2013). On the other hand, the higher frequency of diploids in European localities, indicated in the present study, reflects the intensification of their bisexual reproduction. In any case, the benefits of bisexuality in changing environmental conditions may indirectly indicate the vulnerability of ecosystems.

DNA content and karyotypes of diploid and triploid C. gibelio

The average DNA content in the nuclei of $2n$ and $3n$ *C. gibelio* individuals was similar to that of other 100-chromosome *Carassius* species and *C. gibelio* ($3n = 150$), respectively (Vinogradov 1998; Ciudad et al. 2002).

The karyotype of *C. gibelio* diploids of both sexes equalled that reported for some other populations assumed by Boroń et al. (2011). Similarly, $3n$

females of 150–162 chromosomes (Zhou and Gui 2002; Liasko et al. 2010; Boroń et al. 2011) and $3n$ males possessing from 152 to 156 chromosomes (Liasko et al. 2010; Lusková et al. 2010; Jakovlić and Gui 2011), both with several micro elements are known. Interestingly these extra microchromosomes with repetitive sequences and transposable elements in males are a common feature of sex chromosomes and play a male determination role in *C. gibelio* (Li et al. 2016; Li and Gui 2018). The karyotype patterns given in this study are slightly different compared with other findings in Europe (Boroń 1994; Boroń et al. 2011; Liasko et al. 2010) probably due to many small chromosomes which are similar in size. The diversity of chromosome numbers for *C. gibelio* triploids which is not simply multiples of $3 \times n$ ($= 50$) seems to correspond to their clonal diversity followed by multiple reproduction modes (Zhou and Gui 2002, 2017; Zhang et al. 2015; Liu et al. 2017a). Triploid *C. gibelio* females in European populations generally reproduce gynogenetically, but some interspecific hybrids could also be found (Knytl et al. 2013; Table 1) due to incorporation of genetic material of the spermatozoon into the female pronucleus (Tóth et al. 2005). For this reason, *C. gibelio* is unique among vertebrate species since it can integrate genes of related species, greatly contributing to its adaptability. Anyway, the data from the present and previous studies show that the chromosome number of *C. gibelio* triploids oscillates above 150 and supports the hypothesis of close relations between East Asian and European populations in terms of karyology (Zhou and Gui 2002; Lusková et al. 2010).

Length and weight relationships

Negative allometric growth for triploid *C. gibelio* in the Siemianówka Res. could be attributed to the water quality, e.g. phosphorus concentrations or the trophic state (Tsoumani et al. 2006). Moreover, the growth of *C. gibelio* can vary considerably in the same water body and, apart from sex (present study), might be affected by season, population density, age of sexual maturity, food resources or environmental conditions (Marinović et al. 2016).

The detected differences in the length-weight relationships between sexes (Tsoumani et al. 2006; Liasko et al. 2010; present study) seem not to be the rule (Vetemaa et al. 2005; Šimková et al. 2015). Even though females attain a larger size than males, SL or TL cannot be used to distinguish them, with some exceptions (Liasko et al. 2010). Females, as well as males of different ploidy, did not differ in size, which reflects the fact that in most polyploid animals, larger cell sizes are compensated by their lower number (Leggatt and Iwama 2003). Similarly, the usual triploids growth pattern was not significantly different from that of diploids (Liasko et al. 2011). Although length-weight conversion factors are important in monitoring this invasive species, data from Polish and other European bisexual populations with known sex and ploidy ratios are generally lacking.

Concluding, *C. gibelio* do not have a stable number of chromosomes and their karyotypes varied from 150 to 164, including up to six extra microchromosomes. In the investigated population in Siemianówka Res. there was parity between 2n males and females, and between 2n and 3n females. A relatively high number of mature males were documented, both diploid and triploid, indicating that this population, like some others in Europe, may be undergoing a transition of reproduction mode from unisexual gynogenetic to bisexual reproduction. Our results shown for the first time that the invasion success of *C. gibelio* in Europe seems to be determined by higher incidence of diploid individuals of both sexes, which seem to benefit from reaching stable bisexual populations and can probably be more adapted to a wide range of ecosystem conditions. In comparison, the populations distributed in East Asia are characterized by a higher proportion of triploid individuals of both sexes. Length-weight relationships of *C. gibelio*, calculated for the first time on the basis of a representative number of diploid and triploid females and males, appears not to be ploidy-influenced. Our findings reveal that the presented population is a good model for further study of the potential effects of global warming to temperature-dependent sex determination in *C. gibelio* triploids. This study reports sex ratio and size, and karyotype pattern of *C. gibelio* of different ploidy levels, not only as a component of inland fisheries but also as a common invasive freshwater fish species and a striking example of mating system evolution. So, the presented results contribute to information expanding our understanding on establishment and invasion success of this wide-spread non-native species.

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