Vestnik zoologii, **49**(6): 537–550, 2015
DOI 10.1515/vzoo-2015-0064

DE GRUYTER
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UDC 595.122:597.556.333.7:591.526(262.54)

MORTALITY OF JUVENILE SO-IUY MULLET, *LIZA HAEMATOCHEILUS* (TELEOSTEI, MUGILIDAE), IN THE SEA OF AZOV ASSOCIATED WITH METACERCARIA (DIGENEA)

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Mortality of Juvenile So-iuy Mullet, *Liza haematocheilus* (Teleostei, Mugilidae), in the Sea of Azov Associated with Metacercariae (Digenea). Sarabeev, V. — Age-dependent patterns, including yearly variations of digenean metacercariae infestations of the introduced species, *Liza haematocheilus* (Temminck & Schlegel, 1845), were studied. We evaluated the impacts of three metacercaria species, *Timoniella imbutiforme* (Molin, 1859) Brooks, 1980, *Diplostomum* spp. and *Ascocotyle (Phagicola) longa* Ransom, 1920, on juvenile fish in age from one month to 2+ years old from the Molochny Estuary and neighboring waters of the Sea of Azov by applying Crofton's negative binomial truncation technique, epidemiologic and aggregation indices. Parasite surveys executed in 1997–2014 revealed significant yearly differences in the infection dynamics of studied metacercariae in juvenile fish of *L. haematocheilus*. Metacercariae were absent or fish harboured several times less parasites in 2005–2013 than in 1997–1999. *T. imbutiforme* infection exhibits a convex that was observed in a decline of the parasite load after an initial increase. The infection load of *Diplostomum* spp. increased asymptotically with the fish age reaching maximum value in two years old juveniles. Both the abundance and the prevalence of *A. (P.) longa* were low in juveniles of two month and two years old but relatively high and more or less constant during the rest of the juvenile period. Results of the present study suggest that metacercariae, especially, *T. imbutiforme*, are associated with mortality of juvenile *Liza haematocheilus*.

Key words: *Timoniella imbutiforme*, Molochny Estuary, parasite-induced mortality.

Смертность молоди пиленгаса, *Liza haematocheilus* (Teleostei, Mugilidae), в Азовском море, ассоциированная с зараженностью метацеркариями трематод (Digenea). **Сарабеев В. Л.** — Изучены возрастные и межгодовые особенности заражения интродуцированного вида кефали, *Liza haematocheilus* (Temminck & Schlegel, 1845). Проведена оценка влияния трех видов метацеркарий, *Timoniella imbutiforme* (Molin, 1859) Brooks, 1980, *Diplostomum* spp. и *Ascocotyle (Phagicola) longa* Ransom, 1920, на молодь пиленгаса в возрасте от одного месяца до 2+ лет в Молочном лимане и прилегающих водах Азовского моря, с использованием метода усеченного негативного биномиального распределения, предложенного Крофтоном (1971), эпидемиологических показателей и индексов агрегации. Паразитологические обследования, выполненные в период с 1997 по 2014 гг., показали существенную разницу годовой динамики заражения исследованных видов метацеркарий у молоди пиленгаса. Метацеркарии отсутствовали или зараженность рыб была в несколько раз меньше в 2005–2013, чем в 1997–1999 гг. Динамика зараженности молоди пиленгаса метацеркарией *T. imbutiforme* представлена выпуклой кривой, которая описывается как снижение численности после начального роста. Зараженность метацеркарией *Diplostomum* spp. увеличивается асимптотически с возрастом, достигая максимального значения у двухгодовалых особей. Численность метацеркарий *A. (P.) longa* была относительно низкой в возрасте двух месяцев и двух лет, тогда как в течение остального периода оставалась более или менее высокой. Полученные результаты предполагают, что зараженность рыб метацеркариями, в частности *T. imbutiforme*, обуславливает смертность молоди пиленгаса в Молочном лимане, в результате чего более 50 % молоди, появившейся в Молочном лимане, погибает в течение первого года жизни. Исходя из результатов годовой и возрастной динамики заражения пиленгаса метацеркариями трематод, можно дать следующие практические рекомендации: первое, так как смертность, вызванная паразитами наблюдалась среди высокоурожайных поколений пиленгаса, рекомендуем: а) регулировать количество заходящих производителей на нерест в Молочный лиман, чтобы избежать перенаселения нерестовых акваторий и таким образом уменьшить уровень передачи гельминтов; и б) отлавливать молодь рыб из дикой популяции для их дальнейшего выращивания в аквакультуре, следуя рекомендациям Сабодаша и Семененко (1998); второе, так как зараженность метацеркариями быстро увеличивается с ростом сеголеток, последние должны изыматься из естественной среды в раннем возрасте, предпочтительно в течение летнего сезона, для зарыбления ими морских и солоноватых водоемов.

Ключевые слова:*Timoniella imbutiforme*, Молочный лиман, смертность, вызванная гельминтами.

Introduction

Exploring ecological features of the host-parasite relationships in the environment is a notoriously complicated task (Stanko et al., 2006) and the determination of the parasite-induced host mortality in wild fish populations presents even greater difficulties (Adjei et al., 1986; Ferguson et al., 2011). Dead or diseased fish are rarely found in the field, especially their early juvenile stages, and even if they are, the cause of death can rarely be attributed unequivocally to the parasite impact due to the effects of predation, competitive interactions, environmental pollution etc. (Rousset et al., 1996; Hatcher et al., 2012). However, several techniques have been proposed to assess the influence of the parasitic infection on the host mortality range (Crofton, 1971; Lester, 1984; Adjei et al., 1986). Lester (1984) reviewed the common methods used for detecting mortality due to parasitic infections in wild fishes, many of which require repeatable observations of parasite abundance, prevalence, frequency and aggregation on the same host population. An alternative technique for tracking infections in host populations over time is to compare the tail of the observed distribution to that of the distribution predicted from the first few points of the data, as originally proposed by Crofton (1971). He claimed that there are usually fewer heavily infected hosts in wild populations than would be predicted. An explanation for this phenomenon is that heavily infected hosts are more predisposed to mortality. Although this model was criticized by Mccalum and Dobson (1995), it became widely accepted and is applied extensively in theoretical and empirical models (Adjei et al., 1986; Royce, Rossignol, 1990; Rousset et al., 1996; Galvani, 2003; Ferguson et al., 2011, etc).

Host-parasite relationships are mostly well-balanced in systems with a common evolutionary history, where a host defence mechanism is regularly resisted by the parasite and vice versa. In new host-parasite systems, emerged as the result of a deliberate or accidental introduction, the relationships between native parasites and introduced host or between native host and co-introduced parasites, brought in the new area together with the invasive host species, are unbalanced and unpredictable, as adaptive relations of both parties of the system are broken down. Co-introduced parasites that accompany an invader may host-switch to native taxa, sometimes with devastating results, and also the reverse events may occur when parasites from native host transfer to the invader (Mccalum, Dobson, 1995; Shine, 2012). Lymbery et al. (2014) gathered data on 76 examples of co-introduced parasites that switched to native hosts, in 16 of them information on relative virulence was available. Of these 16 host-parasite records, 14 co-invasive parasites were more virulent in native hosts than in the invader while for the other two cases there was no evidence of any difference in virulence between native and introduced hosts. However, data documenting a negative effect of native parasites on the introduced host, where parasites can serve as important population regulators of invasive host in an ecosystem, are largely lacking (McCallum, Dobson, 1995).

Here, we evaluated the impacts of three digenean metacercariae, *Timoniella imbutiforme* (Molin, 1859), *Diplostomum* spp. and *Ascocotyle (Phagicola) longa* Ransom, 1920, on fry and juveniles of so-iuy mullet (*Liza haematocheilus* (Temminck & Schlegel)) from the Molochny Estuary and adjacent waters of the Obitochna Bay of the Sea of Azovby comparing parasite loads of different age groups of the fish using three analytical techniques: i) comparison of parasite prevalence and abundance between different fish age groups, ii) comparison of parasite aggregation using the variance to mean ratio (VMR) and the exponent k of the negative binomial distribution (NBD) between groups, iii) parasite frequencies analysis in fish using the negative binomial truncation technique developed by Crofton (1971). We also did attempt to estimate the percentage of host population loss as a result of parasite associated fish mortality based on the approach proposed by Adjei et al. (1986).

Liza haematocheilus native to the Amur River estuary and the Sea of Japan was deliberately introduced into the Black and the Azov Seas in 1978. The main goal of the introduction of this fish into the Sea of Azov was seeking ways to increase the fishing capacity of natural water reservoirs and to use abundant and underutilized detritus (Sabodash, Semenenko, 1998). The first self-reproduction of the so-iuy mullet in the new distribution area was reported since 1986 (Yanovsky, Isergin, 1998). The environmental conditions in the Azov-Black Seas appear to be favourable for *Liza haematocheilus* whose growth rate exceeds those of the native mullet species (Okumus and Bascinar, 1997), therefore, this species was considered as prospective subject for the estuarine aquaculture in this region (Starushenko, Kazansky, 1996; Sabodash, Semenenko, 1998). Aquaculture of grey mullets relies on the use of wild seed based on the capture of fry and juveniles in their natural habitats. Such type of extensive aquaculture makes it imperative to acquire the knowledge about appropriate terms of fish capture and epidemiological conditions for the lagoon fish culture (Saleh, 2008).

The introduction history of *L. haematocheilus* in the Sea of Azov has known only one record of the mass mortality of this species in the Molochny Estuary caused by parasites. It occurred in 1996 due to gill microsporidiosis (Maltsev, 1999). According to the results based on all three analytical techniques mentioned above, it is evident that parasites, especially metacercariae of *T. imbutiforme*, have an impact on juveniles of *L. haema-*

tocheilus reproduced in the Molochny Estuary. The fish lost due parasite-associate host mortality may exceed 50 % of the recruitment.

Methods

Study Area, Fish Sampling and Parasite Collection

Juvenile specimens of *L. haematocheilus* were collected by a 10 m long 1–6.5 mm mesh fry dragnets while adult fish were obtained directly from fishermen. This study covers the period of 1997–2014 and gives the analysis of metacercariae infection dynamics in fish stock produced on the Molochny Estuary of the Sea of Azov. Zero year old juveniles were sampled from the canal connecting the Molochny Estuary with the Sea of Azov while fish of older groups from the Molochny Estuary and its adjacent waters of the Obitochna Bay. The present study is based on 319 dissected juveniles of the so-iuy mullet in the age range from one month to two years old and 49 adult fish in age range from 3 to 6 years old that totally represents 12 datasets (table 1). The number of fish typically reached 25–40 fish specimens per sample following recommendations of Gregory and Blackburn (1991), Jovani and Tella (2006), and Marques and Cabral (2007). Of the presented dataset, 3 of 12 samples were in range 17–19 specimens of juvenile fish. Such small sample size also could be used as a reasonable trade-off between not losing too much important information and maintaining acceptable levels of uncertainty (Jovani, Tella, 2006).

Samples of fish from years 1997 and 1999 were matched for comparative analysis to obtain representative samples that was permissible because: a) we were not making direct comparison of the zero year old fish with older groups; b) similar ecological conditions of the Molochny Estuary insured by a certain connection of the estuary with the Sea of Azov characterized the reservoir at those years; and c) high-yield generations of the so-iuy mullet born in the Molochny Estuary recruited stock at that time. The analysis does not include 4 datasets with 26, 51, 46 and 26 individuals of zero year fish sampled in July of 2008, 2010, 2013 and 2014, respectively, because the digeneans were absent in the fish samples. The fish age of zero year old fish was determined in accordance with Sabodash and Semenenko (1998), while the age of older individuals was identified from scale samples (Tsarin et al., 1999). The fish sample in the age designed as one month includes both fry and juvenile fish.

Collected fishes were measured and surveyed for parasites within the day of capture or after holding in a refrigerator during 1–3 days. The muscular tissue, oesophagus, pyloric caeca, intestine, eye lens and internal organs were squashed between two Petri dishes of different diameter and carefully examined under a stereomicroscope for parasites. All helminth parasites were identified and counted. Following the preliminary identification of helminths using the stereomicroscope, selected specimens of larval digeneans were encysted or cleaned of the host tissues (in case of *Diplostomum* spp.) and stained in iron acetocarmine, followed by dehydrating through an ethanol series (from 70 to 100 %), clearing in dimethyl phthalate and mounting as whole mounts in Canada balsam. Taxonomic identification was attempted to the lowest possible level in accordance to Gaevskaya, Gusev, Delyamure et al. (1975), Shigin (1986), Simões et al. (2010). Identification of *Diplostomum* specimens from fish was done to the genus level due to the lack of morphological features useful for identification of larval stages and specific requirements for mounting and examination of the specimens (Shigin, 1986). Adult worms of *Diplostomum* spp. and *A. (P.) longa* were also obtained from wild fish-eating birds of the Molochny Estuary and in the case of *T. imbutiforme* from *Platichthys flesus* (L.) and *Syngnathus typhle* (L.) (Domnich, Sarabeev, 2000).

Data Analysis

For each parasite species, the indices of prevalence and mean abundance in fish were determined according to Bush et al. (1997). The mean abundance was estimated with 95 % confidence intervals (CI) for the comparative reason. The parasite aggregation was characterized by two measures: variance to mean ratio (VMR) and the exponent k of the NBD. A web-based tool provided by Reiczigel and Rózsa (2005) was used to compute the 95 % CI of the mean abundance, VMR value and exponent k and its associated p-value, determining whether or not the parasite data fit NBD.

Comparison of abundance in relation to fish age and yearly dynamics was analyzed by Mann-Whitney paired sample test. This procedure was performed with PAST v3.06 (Hammer, Harper, 2005). The effect of the fish total length on helminth intensity was tested with linear regression technique on previously log transformed data. The relations were considered significant when P-values Pearson correlation were lower than 0.05.

Crofton's truncation technique was applied to determine whether or not digenean metacercariae induce fish mortality. This model involves estimating an overall expected parasite distribution in hosts from the zero and low frequency classes, where lethal effects are less significant (Royce, Rossignol, 1990). Both the observed and truncated distribution curves will fit the NBD, but the truncated curve will fit better because the pattern from the observed data will be missing fish from the high parasite load class, owing to parasite-induced host mortality. If mortality is density dependent, then the truncated curve will show an increase in the number of hosts with higher helminth loads in comparison with the distribution curve of the observed data (Ferguson et al., 2011; Royce, Rossignol, 1990). In the present study the truncation technique was applied to all available juveniles surveyed in 1997–1999. Because the age of fish used in the present study was known, it was possible to compare an estimated pre-mortality distribution from zero year old fish with the distribution of parasites in juveniles of one and two years old. The survival probability of juvenile fish was defined as the ratio of the estimated post-mortality number of fish with x parasites to the expected number prior to parasite-related deaths occurring. Following identification of the survival probability, it is becoming feasible to define the proportion of fish stock and infected hosts that were lost due to parasite infection using formulae 4 and 5 of Adjei et al. (1986).

Crofton's analysis of the parasite distribution was performed using an iterative technique, minimizing χ^2 to determine the best fit of estimated numbers to the observed. It was done by applying the following algorithm: a) estimation of the maximum likelihood value of k from whole data set and work back, truncating sample in small steps; b) evaluation of the mean parasite abundance with formulae of 4.3 of Breyev (1972); c) calculation of theoretical frequencies applying formula of 3.15 of Breyev (1972); d) comparison of the expected distribution from zero to one of the frequency classes with the observed distribution using a χ^2 test. The exponent k of the NBD for this frequency analysis was estimated with a web-based tool provided by Wessa (2015) while Chi-square test was executed with PAST.

Results

Timoniella imbutiforme

Metacercariae were found in skeletal muscular tissues and oesophagus wall of host species. Fry and juveniles of *L. haematocheilus* from the Molochny Estuary were highly infected by *T. imbutiforme* in 1997 and 1999, while in the subsequent years this metacercaria was not found in samples of so-iuy mullet except a single record of this worm in November 2014 (table 1). Analysis of the age dynamics of prevalence and abundance of *T. imbutiforme* shows that infection increased with age, until the fish were at age 3 months and 31–40 mm long. After this, both epidemiologic indexes remained more or less constant for zero year old juveniles. Beginning from one year old juveniles, the infection of *T. imbutiforme* declined and reached minimal values in two years old fish. The parasite survey of adult individuals revealed slightly higher abundance but the occurrence was about twice lower than in two years old juveniles. Mann-Whitney tests revealed the significant difference in the helminth abundance between compared zero year old and one-two years old fish ($U = 4558$, $p < 0.0001$). The regression analysis of relationships between the total fish length and the number of helminths per host shows a similar picture of dynamics indicating significant decrease of parasite intensity with fish length for one-two years old juveniles (fig. 1).

The present observation of aggregation indexes for metacercariae of *T. imbutiforme* revealed the approximately linear relationships between the exponent k and the prevalence ($R^2 = 0.82$, $p < 0.001$), while the VMR is predominantly affected by the mean helminth abundance ($R^2 = 0.4$, $p = 0.07$). The distribution patterns of *T. imbutiforme* in *L. haematocheilus* well fit to the NBD in all surveyed age groups of fish except in age of two month, two years old juveniles and adult fish (table 1). Estimated over-dispersion pattern, measured by the VMR, in zero year old fish, was at least twice higher than that of one-two year old fish. The VMR was influenced by infections in zero year old fish as they

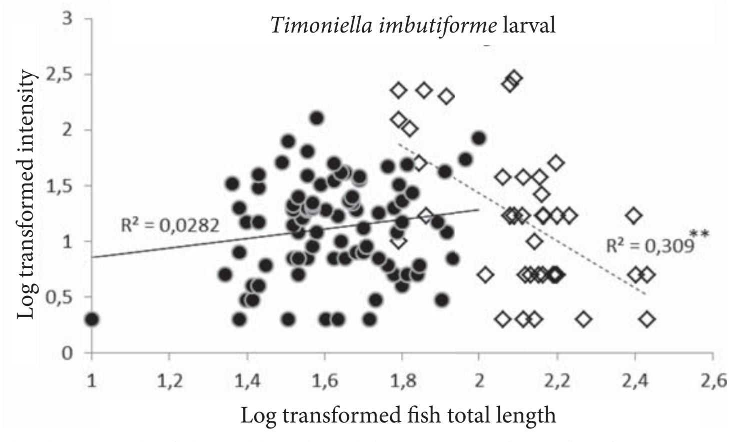

Fig. 1. Relationship between the fish total length and the intensity of *T. imbutiforme* in zero year old (solid line and solid point) and one-two years old (dashed line, open rhomb) of *L. haematocheilus*. **Referred to a significant level of 99 %.

Table 1. **Results of fish and helminths sampling with information on fish age and size, localities, dates, number of studied fish, mean abundance, prevalence and aggregation indices (variance to mean ratio and exponent *k* of the NBD) of *T. imbutiforme*, *A.* (*P.*) *longa* and *Diplostomum* spp. from *L. haematocheilus* in the Sea of Azov**

Host features		Locality*	Month/ Year	n	*T. imbutiforme*				*A.* (*P.*) *longa*				*Diplostomum* spp.			
Age	Total length. mean (range). mm				Mean abundance (CI 95 %)	%	VMR	*k***	Mean abundance (CI 95 %)	%	VMR	*k***	Mean abundance (CI 95 %)	%	VMR	*k***
1 month	26.2 (10–53)	ME	6/ 97–98	37	2.5 (1.16–4.65)	32	11	0.14	–	–	–	–	0.1 (0–0.14)	5	1	n/e
2 months	45.7 (23–75)	ME	7/ 97–99	35	10.3 (5.57–17.8)	37	31	0.11[a]	1.4 (0.31–4.01)	14	16	0.04[b]	–	–	–	–
3 months	45.8 (31–70)	ME	8/ 97–99	38	16.9 (11.3–28.4)	79	35	0.48	2.9 (1.18–7.78)	39	23	0.17	0.7 (0.03–3.46)	8	17	0.02[b]
4 months	56.5 (34–85)	ME	9/ 97–98	19	11.8 (7.32–19)	84	14	0.81	23 (1.47–106)	26	361	0.05[b]	0.3 (0–0.95)	11	3.2	0.06[b]
5–6 months	65.2 (42–100)	ME	10–11/ 97–98	17	23 (13.8–36)	94	23	1.02	3.8 (1.06–9.25)	29	17	0.1[b]	0.4 (0–1.53)	12	5.2	0.06[b]
1 year	100 (62–145)	ME	5–7/ 97–99	18	7.3 (4.56–9.94)	89	5	1.29	2.1 (0.23–4.44)	22	12	0.1[b]	2.7 (1.17–6.37)	39	8.9	0.2[b]
1 year +	152 (122–186)	ME, OB	1–2/ 99	37	1.3 (0.78–2)	41	3	0.40	21.1 (10.2–57)	65	127	0.23	2 (1.03–3.59)	30	7.2	0.14[b]
2 years+	211 (125–270)	ME, OB	8/ 97–99	36	0.7 (0.27–1.29)	22	3	0.16[b]	0.2 (0–0.72)	6	4	0.03[b]	6.9 (3.44–12.7)	39	26.6	0.13
3–7 years	568 (380–715)	ME, OB	5–11/ 98–99	49	1.5 (0.517–3.96)	12	22	0.03[b]	3.1 (0.31–14.3)	12	117	0.03[b]	3.3 (2.04–5.56)	51	10.5	0.3
5 months	76 (30–120)	ME	10/05	34	–	–	–	–	1 (0–3.3)	6	19	0.01[b]	–	–	–	–
5–6 months	86.3 (61–123)	ME	10–11/ 08	25	–	–	–	–	0.3 (0–1.12)	8	5	0.04[b]	–	–	–	–
6 months	83.9 (49–118)	ME	11/14	22	0.05 (0–0.14)	5	1	n/e	2.3 (0.32–7.92)	18	21	0.05[b]	–	–	–	–

* Locality abbreviations: ME, Molochny Estuary; OB, Obitochna Bay.

** It is difficult to interpret *k*, as [a] data does not fit the NBD ($p < 0.05$) and [b] sample too small to check fit of the NBD; *k* = n/e the maximum likelihood estimate of *k* could not be computed due to there are too few data.

had a high mean abundance compared with older juveniles thus increasing the variance of the variable. The exponent *k* of the NBD increased during the first year of life from highly aggregated ($k < 1$) to aggregated ($k > 1$) distribution and decreased in the older age group up to value of 0.03 in adult fish (table 1). The highest values of epidemiologic indexes combined with relatively high values of both the VMR and *k* in juveniles of 5–6 months old may indicate a critical age period when the parasite-induced mortality starts to operate on host population. Both the parasite abundance and the VMR substantially decreased in the following age group category (the 95 % confidence interval of the abundance did not overlap between compared groups, $F = 7.9$, $p < 0.01$ and the VMR in 4.6 times as less in the older group), while the prevalence remained high (89 %) and the *k* even increased from 1.02 to 1.29. This observation points out that highly infected fish is absent in the group of one year old juveniles, although infection is highly prevalent in the population. Thus, it may indicate the presence of the density dependent process operated during the first year of the fish life that resulted in the loss of highly infected hosts due to the parasite-associated fish mortality. In older juvenile fish, the *k* decreased together with all other indexes applied here to quantify the infection rate.

Based on the infection dynamics of *T. imbutiforme* the data set was split in 2 groups, juveniles of 3–6 months, and 1+ –2+ years olds as the pre-mortality and post-mortality periods to conduct the frequency analysis of the parasite distribution. The helminth aggregation is well described by truncated model with the parameters derived from 0 to 4 parasites for juveniles of 3–6 months old and 0 to 2 parasites for young fish of 1+ –2+ years old. The frequency analysis of *T. imbutiforme* predicted that the helminth distribution in both the pre-mortality and post-mortality periods was truncated. The truncation was calculated to occur within the first few infection categories given that the threshold for parasite-associated mortality begins at the presence of 3–4 metacercariae of *T. imbutiforme* per fish (table 2, fig. 2). The truncation point was actually at the beginning of the distribution tail in juveniles of 3–6 months old, while it was higher towards the tail end in fish of 1+ –2+ years old, indicating that the majority of fish had an abundance of infection above the truncation point in the pre-mortality period vs. below in post-mortality (i. e., to the right vs. left side of the curve; see fig. 2 and table 2). The negative binomial curve for 1+ –2+ years old fish is shifted to the lower position, beginning at the level of 3–4 parasites per fish comparing with both the observed and estimated curves of the helminth distribution in juveniles of 3–6 months old (fig. 2). The difference between pre-mortality and fitted post-mortality curves is shown as the survival curve, which declines towards the tail end of the worm distribution on the so-iuy mullet. The proportion of the recruit loss as a result of the infection of *T. imbutiforme* is relatively high and reaches 55 %. For infected fish the estimated loss is slightly higher and counts 67 %.

Ascocotyle (Phagicola) longa

Metacercariae were found predominantly in the oesophagus and intestinal walls, denticulate pharyngeal pad, internal organs and rarely in skeletal muscular tissues of host species. Metacercariae of *A. (P.) longa* were registered in *L. haematocheilus* during the whole period of the fish survey beginning from the second month of fish life (table 1). Both the prevalence and the mean helminth abundance indicate that the parasite load on zero year old juveniles was higher in 1997–1999 comparing to 2005–2014 (Mann-Whitney tests, $U = 3657$, $p = 0.004$). Metacercariae of *A. (P.) longa* were not registered in one month old juvenile fish, cysts of this helminth were found in fish beginning from the second month of their life. Both epidemiological indices were more or less constant in the period from the third month to the age of 1+, when they were relatively high with prevalence reaching 65 % and mean abundance of 23 specimens per host. In the older fish in the age of 2+, the parasite load was significantly lower than in younger fish in age from two month to 1+ ($t = 4.1$, $p = 0.5$). No relationships were found between the total fish length and the number of helminths per host following the regression analysis ($R^2 < 0.05$, $p > 0.05$).

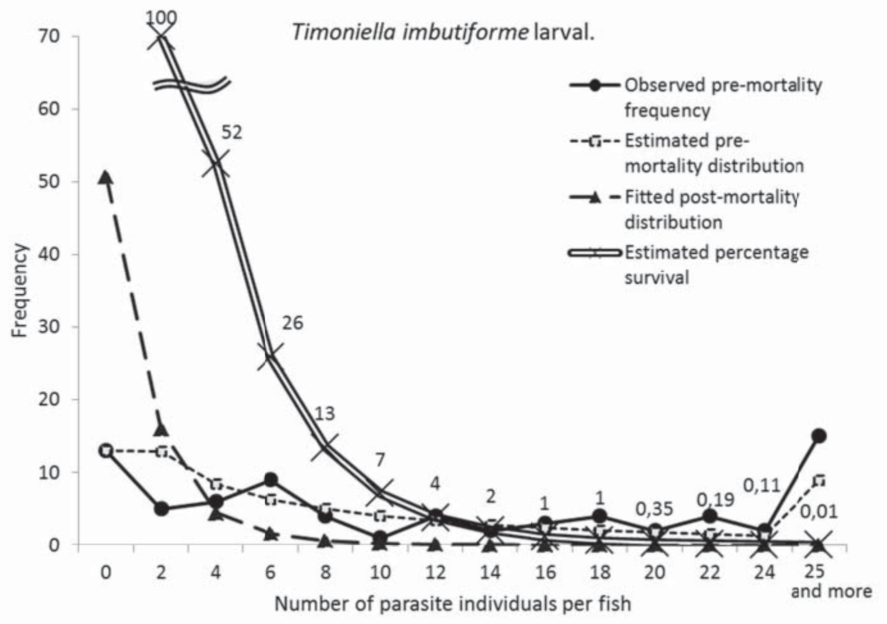

Fig. 2. Frequency distribution of *T. imbutiforme* split following the pre-mortality and post-mortality events. Solid line with solid point and dashed line with open square = the observed and predicted pre-mortality frequency distribution; dashed line with solid triangle = the fitted post-mortality frequency distribution; solid double line with crosses and symbols = the estimated percentage survival of fish with that number of parasites.

There was approximately linear relationships between the exponent *k* and the prevalence ($R^2 = 0.91$, $p < 0.0001$), and the VMR and mean abundance ($R^2 = 0.74$, $p < 0.001$). The age dynamics of the VMR in juveniles was represented by two peaks with the subsequent sharp decrease in its value. Both peaks correspond to the highest mean abundance that declined in the subsequent age group. The VMR increased in adult fish regardless of low mean helminth abundance. The exponent *k* of the NBD was relatively low with a maximum rate not exceeding 0.25 pointing to the highly aggregated distribution of *A. (P.) longa* in *L. haematocheilus*. In other words, it tells us about a relatively low proportion of the fish in the susceptible tail of infection.

Juvenile fish at the age of 3 months to 1+ year old were combined in one data set to apply for Crofton's truncation model because of the even infection parameters in these age groups. The negative binomial parameters were estimated from the fish with 0–45 parasites. The observed frequency of a remained tail was higher than predicted counting five values (table 2) in the range from 58 to 400 metacercariae per host, but this data was not fit for a further categorized analysis. Comparison of parasite distribution between fish of zero-one year old and at the age of two years was not conducted due to the small sample size of the latter age group (table 1).

Diplostomum **spp.**

Metacercariae were found in eye lens of host species. Fry and juveniles of *L. haematocheilus* from the Molochny Estuary were infected by *Diplostomum* spp. in 1997 and 1999, but in the subsequent years this metacercaria was not found in the fish samples (table 1). Metacercariae of *Diplostomum* spp. were recorded in so-iuy mullet beginning from the first month of its life. Two of 37 dissected fish in age about 1 month were infected by a single specimen of *Diplostomum* per capita. Metacercariae were not found in two month old juvenile fish. Beginning from the age of three months the parasite mean abundance steadily increased up to two years old while the prevalence reached the maximum value of 51 % in adult fish. The regression analysis performed on linear measurements of fish and the

Table 2. Frequency distribution of metacercariae infecting juveniles of *L. haematocheilus* from the Molochny Estuary and adjacent waters of the Azov Sea fitted to the truncated negative binomial distribution

No. parasites	Observed	Crofton predicted	No. parasites	Observed	Crofton predicted
T. imbutiforme, 2–6 months old			*A.* (*P.*) *longa*, 3 months–1+ year old		
0	13	13	0	76	76.0
1–2	5	12.9	1–7	28	31.5
3–4	6	8.4	8–14	6	8.3
5–6	9	6.3	15–21	8	4.3
7–8	4	5	22–28	2	2.7
9–10	1	4.1	29–35	1	1.7
11–12	4	3.4	36–42	1	1.2
13–14	2	2.8	43–49	2	0.8
15–16	3	2.4	50 andmore	5	2.3
17–18	4	2.0	*k*	N/A	0.15
19–20	2	1.8	*Diplostomum* spp., 3 months–2+ year old		
21–22	4	1.5	0	126	126
23–24	2	1.3	1–2	10	14.8
25 and more	15	8.9	3–4	4	5.9
k	N/A	0.6	5–6	6	3.6
T. imbutiforme 1+ – 2+ years old			7–8	6	2.5
0	50	50.7	9–10	4	1.9
1–2	12	16	11 and more	9	9.6
3–4	8	4.4	*k*	N/A	0.08
5–6	2	1.6			
7 and more	1	0.7			
k	N/A	0.3			

number of helminth specimens per host further supports the observed infection pattern, revealing positive relationships between fish length and parasite intensity (fig. 3).

The relationships between the VMR, the exponent *k* and the prevalence are approximately linear in *Diplostomum* spp. ($R^2 = 0.87$, $p < 0.001$; $R^2 = 0.89$, $p < 0.01$, respectively), moreover the VMR also moderately correlated to the mean abundance ($R^2 = 0.66$, $p < 0.05$). The distribution patterns of *Diplostomum* spp. in the most samples did not fit well to the NBD in all age groups except the age of two years old (table 1). The estimated over-dispersion pattern measured by the VMR in fry and juveniles was relatively low in range 1–26.6. The VMR reached a maximum value in the fish of two years old, corresponding to the maximum mean abundance. A steady increase in *k* (decrease in aggregation) with age was observed,

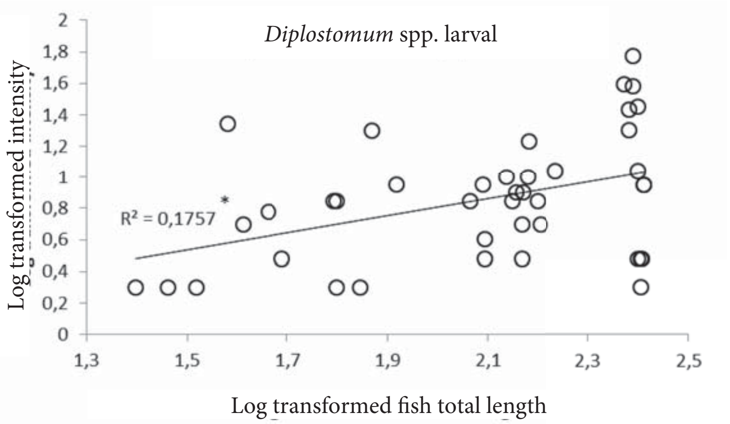

Fig. 3.Relationship between the fish total length and the intensity of *Diplostomum* spp. in juveniles of *L. haematocheilus*.* Referred to a significant level of 95 %.

the lowest value was reported for three month old fish and the highest for the adult group.

Juvenile fish from three months to two years old were selected to execute the frequency analysis. The best fit of the observed data to the estimated frequency was found for the negative binomial parameters obtained from the fish with 0–5 parasites. The observed frequency with 1–4 parasites was less than expected (table 2), but we made the assumption that fish with 5 parasites do not die as the result of their infection. The observed frequencies with 5 and more parasites were very close to or higher than expected. Thus, the truncation model indicated that the parasite-related mortality had not occurred among fish infected by *Diplostomum* spp.

Discussion

Our long-term surveys revealed significant yearly differences in the infection dynamics of studied larval digeneans in fry and juveniles of *L. haematocheilus*. Metacercaria were absent or fish harboured several times less worms (in case of *A. (P.) longa*) in 2005–2013 than in 1997–1999. Probably this is related to the hydrological regime of the Molochny Estuary. Two different stages in ecological conditions could be connected to such drastic difference in the parasite infection dynamics. In the first stage (up to 2002) the Molochny Estuary was characterized by a high level of bioproductivity and biodiversity, as well as by an optimal salinity (17–25 gL–1) insured by a certain connection of the estuary with the Sea of Azov. The second stage initiated by the isolation of the estuary from the Sea of Azov (beginning from 2002). As the result of isolation, the water salinity increased up to levels as high as 30–90 gL–1 while the bioproductivity and biodiversity decreased (Demchenko et al., 2012). For example, species richness of zoobenthos decreased from 88 to 29–30 species (Ivanova, 2006), fish diversity from 36 to 7 species (Antonivsky et al., 2010). Moreover, the bird quantity on the Molochny Estuary was reduced in 3–4 times during the period of the isolation (Demchenko et al., 2012). The mouth of the Molochny Estuary was artificially opened in July of 2014 resulting in revealing of *T. imbutiforme* and increasing both the abundance and prevalence of *A. (P.) longa* in zero year old fish.

The population size of *L. haematocheilus* also strictly depends on the hydrological regime of the Molochny Estuary, with variation from 20 million individuals in zero year old fish in 2007–2011 (Solod, Diripasko, 2012) up to 9 billion in 1992 (Yanovsky, Izergin, 1998). This fish also makes a substantial contribution to the diets of fish-eating birds (Demchenko et al., 1999; Pryahin, 2001; our own observations), for instance, composing about 11 % to the dietary volumes of *Phalacrocorax carbo* L. in the Molochny Estuary (Demchenko et al., 1999). All of these factors indicate that *L. haematocheilus* plays an important role in the transmission of bird parasites, such as *A. (P.) longa* and *Diplostomum* spp. But in the case of *T. imbutiforme* it is difficult to clearly identify the role of the introduced host in the transmission of this worm. The predator species of *L. haematocheilus* is *Sander lucioperca* (L.) in the Sea of Azov (Sabodash, Semenenko, 1998; Pryahin, 2001). All of 22 examined individuals of *S. lucioperca* in 1997–1999 from the Molochny Estuary and adjacent waters did not harbour *T. imbutiforme*, adults of this digenean infect *P. flesus* and *S. typhle* (Domnich, Sarabeev, 2000) but grey mullets seem left out of the diet of these two definitive hosts (Vinagre et al., 2008). Although *T. imbutiforme* is characterized by a wide range of definitive hosts including *P. flesus, S. typhle*, *Dicentrarchus labrax* (L.), *Dentex dentex* (L.) and some gobiid fish (Gaevskaya et al., 1975; Culurgioniet al., 2015; Radujković, Šandić, 2014), only metacercariae of this worm from juveniles of *S. lucioperca* were previously reported (Terekhov, Safrygina, 1981).

The studied here digeneans showed different patterns of age related variations in the prevalence and abundance. Graphical representation of the infection parameters of *T. imbutiforme* dynamics shows a decline of the parasite load after an initial increase of infection. The infection load of *Diplostomum* spp. increased asymptotically with the fish age, reaching a maximum value in two years old juveniles. Both the abundance and the prevalence of *A. (P.) longa* were low in juveniles of two month and two years old while those were relatively high and more or less constant during the rest of the juvenile period. Among the studied metacercaria *T. imbutiforme* was most abundant and prevalent in zero year old fish, followed by *A. (P.) longa* and *Diplostomum* spp. The observed infection dynamics indicate that for the first months of fish life and up to the winter period the metacercaria of *T. imbutiforme* dominated among larval digeneans, but in the subsequent age groups it was replaced by *A. (P.) longa* and *Diplostomum* spp. A number of empirical studies have reported age-infection curves, which either show a continual increase in parasite load with asymptote or a convex dynamics (Quinnell et al., 1992; Krasnov et al., 2006; Musiba, Nkwengulila, 2006; Fergusson et al., 2011).

The accumulation of metacercariae with the age of a host is a typical occurrence (Wilson et al., 2002) that frequently has been attributed to a long life span of digeneans in intermediate hosts (Musiba, Nkwengulila, 2006; Faltýnková et al., 2011). Continuous exposure of the fish to the reinfection throughout the life, their inability to develop age related immunity, and longevity of metacercariae could be an explanation of the increase in the worm load. Metacercariae of *Diplostomum* spp. can live for up to four years in fish and perhaps until their host dies (Valtonen, Gibson, 1997). Longevity of other metacercariae can be shorter than that of *Diplostomum* spp., their life expectancy is limited by age of 18–30 months (Mitchellet al., 2011) or by other data could not exceed 8–10 months (Faliex, 1991). Although lacking information about the life span of *A. (P.) longa* and *T. imbutiforme* and so absence of clear evidence on their life expectancy, we should state that there were no dead metacercariae recorded in juvenile fish during the present study. At the same time the presence of dead encysted metacercariae in adult fish of *L. haematocheilus* was a common observation. The water temperature in the Molochny Estuary and adjacent waters is low, not exceeding +2 °C during winter period from December to March with minimal temperature reached –1.5 °C during cold winters (Matishovet al., 2008; Directory…, 2006), that must surely further extend the longevity of metacercariae.

Among studied here host-parasite systems relationships between juveniles of the so-iuy mullet and different parasites, relationship with *Diplostomum* spp. was found to be not associated with the mortality risk of any component of the system by several reasons. First, the infection level of *Diplostomum* spp. was relatively low in *L. haematocheilus* with maximum intensity reached 58 metacercaria per fish in the age of 2+. Experimental studies on the pathogenicity of *Diplostomum* species revealed a reduced optomotor and visual response in fish infected by more than 100 and 25 metacercariae in a lens, respectively (Ieshko, Shustov, 1982). Actually, only 6 of 237 (or less than 3 %) studied fry and juvenile fish of the so-iuy mullet in 1997–1999 harbored more than 25 metacercaria in 2 eyes. Second, the worm aggregation, abundance, and prevalence increased with the age of host for the juvenile period of the life of *L. haematocheilus.* Such pattern of parasite load and dispersion can occur when the host does not acquire either immunity or defensive behavior with age and the rate of acquisition of new parasites would exceed the rate of parasite mortality due to host defense (Krasnov et al., 2006). Finally, the frequency analysis of the parasite distribution did not reveal density dependent parasite-induced host mortality in this host-parasite system of *L. haematocheilus* and *Diplostomum* spp. that further supports the assumption on an absence of mortality risk.

T. imbutiforme was indicated to be associated with mortality of juvenile fish. As pointed above the worm load of *T. imbutiforme* in *L. haematocheilus* declines after an initial increase. There are a number of mechanisms that might account for such age dynamics of the parasite load. These include parasite-induced host mortality, host-induced parasite mortality, acquired immunity and behavioural changes in older fish (Rousset et al., 1996; Wilson et al., 2002; Musiba, Nkwengulila, 2006; Stanko et al., 2006). Both the frequency analysis of parasite distribution and the age dynamics of the parasite aggregation indicate the parasite-induced host mortality occurring in the host-parasite system of *T. imbutiforme* and *L. haematocheilus*. The decline of the worm abundance and the aggregation with a remaining high level of prevalence observed for fish at the age of one year indicates that parasites were lost due to mortality of heavily infected hosts. During the juvenile stage of fish life, the distribution of *T. imbutiforme* shifted from highly aggregated patterns to a low level of aggregation. A general characteristic of parasite distribution is that most host individuals harbouring low numbers of parasites, but a few individuals playing host too many, so parasite distribution among hosts is characterized by a high level of aggregation (Wilson et al., 2002; Krasnov et al., 2007). Crofton (1971) found that the greatest control by the parasite species occurred when *k* of the NBD was in the range between 1 and 3. Our results are consistent with Crofton's views revealing the parasite-induced mortality in fish age groups when *k* of the NBD for *T. imbutiforme* was higher than 1.

The predicted threshold of parasite-associated mortality for *T. imbutiforme* was approximately 3–4 metacercariae per fish in the pre-mortality period. Most studies using Crofton's truncation technique only provide a prediction of the number of heavily infected hosts that should have theoretically existed before death (Royce, Rossignol, 1990). Because the fish age was known, we were able to compare the pre and post-mortality distribution of *T. imbutiforme* in juvenile fish of *L. haematocheilus*. Results of this comparison are consistent with the previous assumption, indicating that parasite-induced host mortality may occur even in lightly infected fish, as the post-mortality fitted curve lowers out beginning at the level of 3–4 parasites per fish. Wild populations typically have only a small percentage of hosts that occur above the truncation point and hence parasite-associated mortality seldom threatens an entire population (Ferguson et al., 2011). Most of fish in the age range of 2–6 months old (67 %), however, were below this threshold level indicating that the largest portion of zero year fish population was affected by *T. imbutiforme*. The threshold for parasite-associated mortality indicates the level of infection where mortality begins to occur, but due to the dynamic nature of the process not every fish will die from infection at the tested time point (Adjei et al., 1986; Ferguson et al., 2011). Nevertheless, analysis of the pre and post-mortality distribution of metacercariae in juvenile fish indicates that 55 % of the fish stock of *L. haematocheilus* dies during a year beginning from December. This is reflected by the absence of heavy infections in 1+ –2+ year old fish. In comparison, there were only 3 of 73 (4 %) fish in age of 1+ –2+ year old with infections above this level, suggesting that fish surviving to this age are significantly less presented among zero year old juveniles.

Age-related behavioural changes in older fish also may affect a metacercaria burden in *L. haematocheilus*. Fry and early juvenile fish prefer shallow waters while older juveniles migrate to deeper waters that prevent infection by the metacercariae. The other behaviour change is juvenile spillover from the Molochny Estuary to neighbouring waters of the Obitochna Bay of the Sea of Azov, where fish find comfortable temperatures and oxygen in deeper waters of the Sea of Azov. Our samples reflect this pattern of the fish behaviour for older juvenile age groups (table 1). Mixed samples may affect results of the analysis, because those could potentially represent mixed fish stock, but we estimate such probability to be a rare event for 1997–1999. Naturalization of *L. haematocheilus* in the Sea of Azov is closely related with the Molochny Estuary where the first high-yield natural generations were born at the end of 1980th and beginning of 1990th (Yanovsky, Izergin, 1998; Sabodash, Semenenko, 1998; Solod, Diripasko, 2012). In the middle of 1990th eggs and yearly-juvenile stages were registered in the Sivash Lake and the Taganrog Bay (Yanovsky, Izergin, 1998; Nadolinskiy, 2008). But the effectiveness of reproduction and the stock recruitment of the so-iuy mullet in the Sea of Azov remain to be recognized in the Molochny Estuary thanks to its optimal salinity for the larval development (Solod, Diripasko, 2012).

A. (P.) longa was also indicated to be associated with host mortality but much less so than *T. imbutiforme*. Our study of the age dynamics of *A. (P.) longa* revealed two peaks with the subsequent sharp decline in both the epidemiologic parameters and the over-dispersion pattern. The truncation model indicated that juvenile fish with at least 45 metacercariae per host can survive. These observations indicate that some mortality may occur in juvenile fish, but it affects only heavily infected fish, that did not exceed 4 % of all. Highly aggregated distribution of *A. (P.) longa* in *L. haematocheilus* requires more data to evaluate results of the truncation analysis.

The parasite-related mortality in juveniles of the so-iuy mullet is most likely indirect. Parasite infection was found to affect host condition and survival rate (Francová, Ondračková, 2013). Few zero year dead fish were found floating along the water's edge (about 6–8 fish per 100 meters) near Kyrylivka village at the end of November 1998 after the first frosts. Combination of high winds and shallow water can alter water temperatures as much as 3 °C within a day (Bolshakov, 2013). Examined 15 dead fish were infected by *T. imbutiforme* in a range from 11 to 75 metacercariae per host. These fish were also infected by other parasites common to the fish age (Sarabeev, Domnich, 2000) that prevents conclusion about mortality source. No inflammation or malformations were detected in dead fish indicating that parasite infections might reduce fish survival in case of the environmental stress. Age and temperature-related die-offs have also been shown in other studies of fish parasites (Khan, 2009). Another key factor affecting fish stocks in the Molochny Estuary is the oxygen depletion that may occur at winter due to ice covering and summer as result of high temperature and decomposition of plant residues. The oxygen depletion was also considered as a cofactor affecting mass mortality of *L. haematocheilus* in the Molochny Estuary that occurred in 1996 (Demchenko, 2004). The mortality rate of infected fish is always much higher than uninfected due to predation (Lafferty, Morris, 1996). The Molochny Estuary is an important area for both breeding and migrating birds (Directory… , 2006) and since 2010 it is included in Pryazovskyi National Wildlife Park that could provide a source of various fish-eating birds to prey upon fish in the estuary.

In conclusion, we have shown that the relationships between host age and parasites are highly complicated and involve the interplay of several processes. These processes can include the local variation in environmental conditions (yearly and seasonal changes in temperature, salinity, dissolved oxygen concentration), the richness and abundance of local assemblages, the host resistance (behavioral defense) and mortality. Our results suggest that metacercariae, especially *T. imbutiforme*, are associated with mortality of juvenile so-iuy mullet. However, whether this parasite associated mortality is additive or compensatory remains under a question mark. We stress the importance of parasites as a limiting factor for the introduced population of so-iuy mullet in the Sea of Azov. The current spawning area of *L. haematocheilus* includes the Taganrogsky Bay, the Sivash Lake and the lower reach of the Don River (Yanovsky, Isergin, 1998; Solod, Diripasko, 2012) and our analytical techniques could be applied to these water areas. Important practical implications can be derived from the results of the present study. First, as parasite-related mortality observed among high-yield stocks of *L. haematocheilus*, there is reason a) to regulate a quantity of the spawner stock in the Molochny Estuary to escape overcrowding of the spawning area and decrease the parasite transmission rate, and b) to catch juvenile fish from wild population for their further growing in aquaculture following recommendations of Sabodash and Semenenko (1998). Second, as the metacercaria infection rapidly increased in zero year old juveniles those should be removed from natural environment in early juvenile age, preferably in the summer season, and reintroduced in marine or brackish reservoirs.

We are thankful to Ievgen Tkach for his help in sampling and dissecting fry and juvenile so-iuy mullet in 2007–2014. The present study is benefited by financial support from ELECTRA scholarship #1300424, Erasmus Mundus Action 2.

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Received 5 October 2015
Accepted 29 October 2015