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HEALTH PROFILE OF *ALOSA IMMACULATA* (BENNET, 1835) DURING ITS SPAWNING MIGRATION IN THE DANUBE

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Abstract

This study characterizes the haematological and parasitological profiles of the Pontic shad during its passage in the freshwater environment (Danube, Mm 74-77) as part of the reproduction migration. The investigations were carried out by classic methods on specimens captured during the 2011 and 2018 migrations. The obtained values for the haematological parameters, in the both samples sets, varied in the following ranges: red blood cells count (RBCc) 1.40÷3.82 (×10^{6}cells/µL), haemoglobin (Hb) 9,29÷16.87 (g/dL), haematocrit (Ht) 32,75÷88.00 (%), mean corpuscular volume (MCV) 146,37÷298.47 (fL), mean corpuscular haemoglobin (MCH) 38.32÷80,33 (pg), mean corpuscular haemoglobin concentration (MCHC) 15.14÷34,95 (g/dL). From parasitological point of view, in the analysed Alosa immaculata, the most frequently encountered parasitosis was produced by Contracaecum aduncum, in direct correlation with the shad's diet. The prevalence of the infestation with this nematode was quite high in both data samplings. Extending the future research regarding the specie's health condition at different times of migration, from Black Sea till Danube upstream, can complete the profile of the physiological reactions of this species with great ecological importance.

Key words*: Alosa immaculata, condition, haematology, health, parasitology.*

INTRODUCTION

In-depth knowledge of the biology of the Pontic shad (or Danube shad) *Alosa immaculata* (Bennet, 1835), a specific migratory species of the Ponto-Caspian basin together with sturgeons, has become of increased interest for Danube's riparian countries, considering its ecological relevance due to transition between marine and freshwater environments and ability to cope with the challenges associated to these habitats' heterogeneity (Ciolac, 2004; Višnjić-Jeftić et al., 2013; Dobrin et al., 2013; Năvodaru and Năstase, 2014; Rozdina et al., 2015). These studies are scientifically interesting, highlighting the great value of the migratory fish species as tools for researching the function of the habitats interconnection during fish life cycle and the ecosystems overall health, diversity and conservation (McDowall, 1997; McIntyre et al. 2015; Tamario et al., 2019), as well as for understanding the patterns in fish behaviour plasticity or phenotypic flexibility, too (Bernatchez and Dodson, 1987; Griffths, 2006; Bloom et al., 2018).

The adults of the Pontic shad, after they have grown and reached sexual maturity in the marine environment of the Black Sea, return in the Danube River for reproduction, early in spring. This anadromous movement of the Pontic shad starts under the influence of some environmental cues like the thermal regime of the water and, especially, by the intensity, amplitude and duration of the spring floods discharged in the river mouths (Năvodaru, 1997; Năvodaru and Waldman, 2003; Ciolac and Patriche, 2004; Rozdina et al., 2010). So, this species presents a physiological plasticity that allows it to cope, simultaneously, with the environment's change, the completion of gonad maturation for spawning, energy consummation for upstream swimming, as well as with the modifications into the feeding regime and parasitological profile during migration.

The latest published data indicate an increased decline of the Pontic shad stocks, suggesting potential issues in the conservation status due to anthropogenic pressures such as water pollution (Višnjić-Jeftić et al., 2010), lower water level (Năvodaru and Waldman, 2003), overfishing mainly in brackish waters from the Danube River mouth area where fish are more exposed waiting for the changeover in their osmotic regulating systems from saline to freshwater.
For maintaining the physiological balance, the organism mobilizes his resources and regulatory mechanisms and, among these, the blood carries a primary homeostatic role. The reaction of the blood's constituents consists in qualitative and quantitative changes reflecting the homeostasis possibilities and the health status (Cristea and Munteanu, 1995; Witeska, 2013; Maceda - Veiga et al., 2015).
In order to know more about the physiological reactions of this species, the present paper brings new data, additionally to those reported in a previous study (Grecu et al. 2018), for describing the blood features in relation to shoal shads' fitness and parasitological status associated to each sampling period.

MATERIALS AND METHODS

The collected specimens of Pontic shad were captured for scientific purposes during the spawning migrations along Cotul Pisicii area (Mm 74), in the same period, respectively first decade of May in 2011 (n=18) and 2018 (n=22). Almost 2 mL of blood samples were collected into tubes containing lithium heparin as anticoagulant, *in situ*, immediately after the catch, by excising the caudal peduncle, carefully avoiding the water contact, and transported at 4-8ºC for haematological analyse in the labs. After blood sampling, the shad specimens were transferred, individually, in polyethylene bags for parasitological study. Further, all the analyses were carried out within the Research Centre MoRAS-UDJ Galati (https://erris.gov.ro/ROMANIAN-CENTER-FOR-MODELLIN).

Fish condition

Each shad specimen was measured for the total length (Lt, cm) at a precision of 5 mm, weighed to an accuracy of 1 g scale for total weight (Wt, g) and fish condition was assessed by the length-weight relationship (LWR) $W=a \times L^b$ (Jones et al., 1999). The analyse of the growth rate through this indicator by determining the exponent "b" (regression slope) and it's difference from isometric value of "3.0" indicates the direction of condition change.

Haematological analysis

The blood was analysed within 4 hours after the sampling by routine methods used in fish haematology: the red blood cells count (RBCc, x 10^6 /µL) was determined on Neubauer improved hemocytometer at a magnification of ×400 using Vulpian diluting solution. The haematocrit (Ht, %) was determined with microcapillary tubes centrifuged for 5 minutes at 12000 rpm. The haemoglobin concentration (Hb, g/dL) was spectrophotometrically determined at 540 nm wavelength applying cyanmethaemoglobin method. Using standard formulas, the red blood indices were calculated: the mean corpuscular volume (MCV), the mean corpuscular haemoglobin (MCH) and the mean corpuscular haemoglobin concentration (MCHC).

Parasitological analysis

The sampled fish were examined for ectoparasites and endoparasites using standard parasitological procedures. The taxonomic classification and identification of the parasites were done according to Munteanu (2005) and Bauer (1984, 1985, 1987). Areas around the fins, nostril, operculum and the buccal cavity were examined for external parasites (monogeneans and crustaceans). Each fish was opened dorso-ventrally and its internal organs were examined for parasites. The entire digestive system was removed and placed in a Petri dish with physiological saline solution. Observations on parasites were performed under stereomicroscope or classic microscopy using the AXIO-Imager A1 (ZEISS) microscope connected to a CCD camera AXIOCAM MR3 for images capture. The usual indicators (prevalence, intensity level, mean intensity) were used to express the infection level after Bush at al. (1997) excepting the intensity of parasitism that was assessed using an arbitrary scale in function of the parasite density into the microscopic field: low (less than 5 parasites identified in a microscopic field); medium (5 to 10 parasites), high (more than 10 parasites).

Statistical analysis

The obtained data were statistically analysed for normal distribution using Kolmogorov-Smirnov test ($p>0.05$, K-S test) and independent t-test ($p>0.05$, t-test) from SPSS Statistics 17.0 program, and descriptive statistics from Microsoft Excel 2016 program. Also, the data heterogeneity was assessed by computing the variation coefficient (CV, %) and the results were presented as mean ± standard deviation (M±SD) for haematological data and mean ± standard error (M±S.E.) for parasitological data.

RESULTS AND DISCUSSIONS

Fish condition

The series of data analysed for each year showed no deviations from the normal distribution ($p>0.05$, K-S test) that permitted us to apply furtherly the parametric tests. Statistics reveals significant differences in Pontic shad size between years (Table 1). Migration sampling from 2011 consisted of shads between 29.00 to 34.50 cm long in size and 210-326 g weight that correspond, according to Năvodaru and Waldman (2003), to a dimensions structure associated to 3- and 4-years old shads. In comparison, the sizes of the 2018 individuals ranged between 23.5 to 30.00 cm length and 111 to 198 g weight that correspond to younger shads, belonging to the 2- and 3-years classes. The obtained equations for LWR in each migration present different "b" coefficients indicating allometric growth ($b\neq3$) but still ranging in the limits determined by other authors (Table 1). Monitoring the LWR in a fish population, for longer periods, based on old and new data gathered over the time, is considered a simplified system in assessing the fish welfare in wild population (Bolger and Connolly, 1989; Jones et al., 1999) because they indicate the tertiary or whole-animal responses to stress (Barton et al., 2002) with relevance in fish population management under consideration. Basically, in the conventional fisheries model, values of $b<3.0$ indicates a decrease in condition or elongation in form, $b>3.0$ indicates an increase in the condition because the increase in weight exceeds the increase in length. The larger the difference from 3.0, the higher change in the condition could be observed. Yilmaz and Polat (2011) found no significant difference in the slopes of Pontic shad females and males within season, meaning that the female and male individuals have the same body shapes in the same seasons. Therefore, the fact that the b value calculated for 2018 sampling is lower than that the 2011 value, for the same season period of sampling (beginning of May), could be attributed to a poorly nutritional status of the 2018 shad individuals from our catch. A possible constraint in our results is related to the small number of specimens used for condition determination. This condition factor is influenced by internal factors like diet, disease, reproductive status or by those external including season, site binding conditions, chemical exposure.

Table 1. Biometrics of the analysed Pontic shads

Statistical parameters	W_t (g)		L_t (cm)		Length-weight relationship (LWR))	
	2011	2018	2011	2018	2011	2018
M±SD.	266.71±39.20[a]	140.86±24.44[b]	31.96±1.55[a]	26.11 ±1.70[b]	$W = 0.0182 * L^{2.7674}$ $R^2 = 0.8483$ b=2.7674	$W = 0.042*L^{2.4856}$ $R^2 = 0.9388$ b=2.4856
CV (%)	14.70	17.35	4.85	6.51		
Năvodaru and Waldman (2003)					$W = 0.0563*L^{2.457}$ b=2.457	
Rozdina et al. (2013)					$W = 0.0234*L^{2.7315}$ $R^2 = 0.95$, b=2.7315	
Năvodaru and Năstase (2014)					$W = 0.0142*L^{2.8735}$ $R^2 = 0.8442$, b=2.8735	
Ibanescu et al. (2016)					$W = 0.0526* L^{2.487}$ $R^2 = 0.85$, b=2.487	

Different letters in a row indicate significant differences between data series for the same variable ($p < 0.05$; independent t-test)

Haematological profile

The statistical analyse of the blood data series presents normal distributions (p>0.05, K-S test) allowing the application of parametric tests.
The overall data table of the Pontic shad blood parameters shows high values (Table 2), in correlation with the specie's natural motility. The pelagic Pontic shad which actively migrates upstream in the Danube for spawning is an anadromous members of the family Clupeidae. Generally, all the family members are considered comparatively strong swimmers (Clough et al., 2001).
For American shad (*Alosa sapidissima* Wilson, 1811), Leonard and McCormick (1999) concluded that its performance and migratory success is increased by the changes in haematological physiology that occurred during upstream swimming.
In our studies, the RBCc indicates a range of variation from 1.40 to 3.82 (x10^6 cells/µL), highlighting the intense process of erythropoiesis required to cover the high oxygen uptake necessary to such an aerobic exercise.
The comparison of the RBCc data shows a significant difference between the years: the smaller values of the 2018 shads could be explained by the dominance of the younger individuals (2 years age) and by the inferior status of the shad's condition.
Similar RBCc values were indicated by several authors: 3.0÷4.2 x10^6 cells/µL (Soldatov, 2005), 3.0 x10^6 cells/µl (Stancioiu et al., 2006) for pelagic actively migrating species. The increase of the erythropoietic rate in order to maintain the level of functional activity of the organism could also explains the release of juvenile erythrocytes like polychromatophilic normoblasts found into the circulating blood of 2011 specimens, in a percentage of 6.09 ± 1.19 % (Grecu et al., 2018).
The Ht is an accurate blood analyse parameter and considered a valuable primary diagnostic index for fish health. Our data show a positive and direct correlation of high Ht values with the increased RBCc explained above. The range of 2011 Ht values varied between 48-88 % and that of 2018 from 32.75 to 61.4% maintaining the same statistic difference between the samples. These high values, over the known amplitude for majority fish species that ranging from 20% to 45% (Hrubec & Smith, 2010), are comparable with those published by Leonard & McCormick (1999) concerning the American shad. In their study, the authors found significant difference biased by sex (60±0.5 % for males and 54±0.7 % for females) with a clear Ht elevation tendency along the upriver sites. Jawad et al. (2004) found for Indian shad (*Tenualosa ilisha* Hamilton, 1822) a positive and direct relation between haematocrit values and the length of the fish under a polynomial equation. Also, the haematocrit of herring (*Clupea harengus* Linnaeus, 1758) was found positively correlated with body length and temperature (Everaarts, 1978). A similar tendency could be observed on our data, too.
The Hb is considered the most direct measure of oxygen carrying capacity (Huston, 1997; Witeska, 2013), reflecting the aerobic swimming ability of the fish. The high values determined for this parameter (11.76-16.10 g/dL in 2011; 9.29-16.87 g/dL in 2018) indicate that the members of this species are capable of sustained locomotor activity for fulfil the migration process by upstream swimming, without been influenced by their condition status or age. From this point of view, no statistical difference between years was determined. Higher haemoglobin values were also reported for American shads, increasing progressively, during their upriver migration (Leonard and McCormick, 1999).

Table 2. Haematological parameters of the analysed Pontic shads

Haematological parameters		**RBCc (x10^6 /µL)**	**Ht (%)**	**Hb (g/dL)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dL)**
2011	M±SD	2.85 ±0.57 ^{a}	58.42±8.71 ^{a}	13.97±1.15	211.53±44.28	50.96±11.21 ^{a}	24.24±2.92 ^{a}
	CV (%)	20.10	14.91	8.23	20,93	22.00	12.06
2018	M±SD	2.42 ±0.47 ^{b}	50.62±6.96 ^{b}	14.17±1.74	213.64±32.81	60.04±10.02 ^{b}	28.19±2.84 ^{b}
	CV (%)	19.42	13.74	12.28	15.36	16.69	10.07

Different letters in a column indicate significant differences between data series for the same variable (p < 0.05; independent t-test)

Based on the found haematological parameters, the erythrocyte's indices were further determined, the results reflecting the state of physiological effort and featuring the transport capacity of the erythrocyte in oxygen.
For the MCV, data statistics shows no significant difference between the average annual values. However, the reference range can be greatly extended taking into account the variation limits of the values range (154.45-29.47 fL in 2011; 146.37-277.26 fL in 2018).
The MCH indicates a significant high load with haemoglobin of the erythrocyte for 2018 shads (40.10-80.33 pg) in comparison with those from 2011 (38.32-73.16 pg).
The MCHC varies significantly between years due to differences in size of circulating RBCs. The average value of MCHC in 2018 indicate that the erythrocytes were loaded with a higher amount of haemoglobin (28.19 g/dL), meaning that almost 28% of the erythrocyte volume consists of haemoglobin that carries oxygen to the tissues (23.21-34.95 g/dL) in comparison to 2011 (15.14- 28.14 g/dL).
The data assessment of the erythrocyte's indices shows slightly higher values for 2018, possible indicating the presence of more mature red cells into the circulating blood of 2018 shads. Hrubec and Smith (2010) explained that higher MCV, MCH, and MCHC could be found in older cells due to increase in size and haemoglobin content with time during RBC development.

Parasitological profile

Four species of parasites belonging to 3 systematic groups were identified (Table 3) in both analysed samples of *Alosa immaculata*. The prevalence of total parasite infestation showed the maximum degree of 100 % due to the fact that all the specimens were contaminated with at least one kind of parasite.
The highest prevalence of parasitic infestation belonged to the nematode *Contracaecum aduncum* (syn. *Hysterothylacium aduncum* Rudolphi, 1802) which was found into pyloric caeca, stomach and intestine of 94.44 % of specimens in 2011 and 95.45% in 2018, respectively (Figure 1). The worm burden in 2011 was quite higher in comparison with 2018. This nematode is a typical marine parasite that can only reach freshwaters along with hosts that migrate from the sea to rivers due to the diet spectrum of the shad, mainly composed of crustaceans (Mysidae, Gammaridae etc.) and small fish species which are the primary intermediate hosts for those intestinal nematodes (Munteanu and Bogatu, 2005).
The monogenic worm *Mazocreas alosae* (Hermann, 1782), a specific parasite of the Pontic shad (Figure 2), was determined as the next most common infestation after was isolated in almost 2/3 of specimens even if the presence of this parasite on the shad gills was considered low.
The presence of *Allocreadium isoporum* (Looss, 1894) in the intestine of affected fish (fig. 3) was assessed only in the 2018 samples and was observed an extreme dynamic among individuals, from 2 to 57 parasites/host. Also, only 2 specimens of another digenean trematode were found in the intestine of one shad during 2018, assigned after morphological features as, most probably, *Coitocoecum skrjabini* (Ivanitzky, 1928).
During 2018 sampling examination, traces of the marine pollution by plastics were found in the form of particles ingested and present in the terminal blind sac of the shad stomach (fig. 4). It was a clear proof of the magnitude scale in aquatic pollution by plastics and the fact that it became a very worrying problem of nowadays.

Table 3. Species of parasites identified in the analysed Pontic shads

Systematic group	Species of parasite	Organ/area infested	Prevalence (%)		Parasitic intensity (level)		Mean intensity (M±S.E.)	
			2011	2018	2011	2018	2011	2018
Monogenea	*Mazocreas alosae*	gills	66.67	72.73	Low	Low	4.17±0.67	5.63±0.95
Trematoda	*Allocreadium isoporum*	intestine	-	31.82	-	Medium	-	11.00±7.70
	*Coitocoecum skrjabini**	intestine	-	4.55	-	Low	-	2.00±0.00
Nematoda	*Contracaecum aduncum*	pyloric caeca, stomach, intestine	94.44	95.45	High	High	55.76±7.65	32.38±6.88

*see explanation in the text

Figure 1. General view of *Contracaecum aduncum* attached to the stomach tunica

Figure 2. General view of the monogenean *Mazocreas alosae* detached from the Pontic shad gills

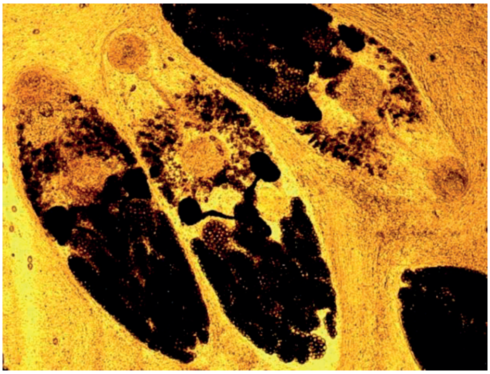

Figure 3. Digenean *Allocreadium isoporum* into the intestinal mucosa of the Pontic shad (x 100)

Figure 4. Plastics particles present into the shad's digestive tract

CONCLUSIONS

Phylogenetically, the migrations of the diadromous fish were caused by various reasons as a life strategy for species survival. In present-day, anthropogenic pressures have seriously impacted the migration pathway continuity or the habitats quality, with unpredictable results that will affect the migration benefit itself.

The Pontic shad *Alosa immaculata* is a typical diadromous fish species with a long-distance locomotor activity from marine saltwater to freshwater as adults and a passive drift for shad's larvae from Danube River towards the Black Sea. Specialists with interest in fisheries management are looking for more information about shad biology that are necessary for species conservation and responsible stocks management.

Any information on physiological, biochemical or behavioural reactions during the migratory activity of the species can bring valid answers on the ability of the fish to cope and acclimate to environmental fluctuation without being forced beyond its limits.

Our goal was to continue the investigation of the Pontic shad patterns in its physiological flexibility for maintaining welfare status during spawning migration. In the condition of our size sampling constraint, data regarding shad condition reflects a small decrease in shad fitness between 2011 and 2018 suggesting the onset of physiological stress during the 2018 migration, with younger age class dominance.

The Pontic shad samples showed an intense erythropoiesis process (ranges with extremely high values of RBCc, Ht and Hb) explaining the high blood oxygen carrying capacity of the species, as is expected in such energetic effort imposed by upstream swimming in Danube River during high flows springs. Our comparative data between 2011 and 2018 exhibited the dependence between haematological variables and fish condition and, possible, fish age.

From parasitological point of view, the analysed *Alosa immaculata* showed a massive infestation with intestinal nematodes, in both sampling, this aspect being related to the feeding regime at the beginning of migration.

Our study results regarding the haematological and parasitological features of the Pontic shad enlarge the biological database of this species, being useful to evaluate the quantitative and qualitative changes that may occur in the alteration of organism' equilibrium state. Future research on Pontic shad should find how its organism regulates the blood's osmotic pressure during the transition from sea to freshwater and other physiological responses during environmental change. These features correlated and integrated with studies regarding genetic diversity in the shad population spur future applied research for species protection.

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