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## A pilot study of two Lower Danube sturgeon species offspring diversity in a feeding area community

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**Abstract:** Current knowledge about sturgeon behavior is incomplete and certain aspects of it are completely unknown, despite tremendous conservation efforts. This paper describes aspects of annual recruitment, fitness distribution and genetic diversity of the offspring of two sturgeon species – *Huso huso* (beluga sturgeon) and *Acipenser stellatus* (stellate sturgeon). To ensure a step forward in understanding their behavior in general and offspring ethology in particular, we conducted a pilot study on the 2016 sturgeon offspring diversity in the Lower Danube River, on the rkm 123 feeding area community. Our approach allowed us to draw a hypothesis about offspring behavior that can also be applied in other feeding areas on the Danube River. Namely, from the convergence of the morphological and genetic data, we found that the two studied sturgeon species have distinct and complex downstream migration behavior. Overall, the present paper presents a pilot study in sturgeon behavior that reveals several traits of offspring ethology. However, further analyses are needed to decode the cryptic behavior of sturgeons.

**Keywords:** Lower Danube, *Huso huso*, *Acipenser stellatus*, young of the year, diversity, feeding area

### INTRODUCTION

The sturgeons represent an extremely valuable natural heritage of the Danube River Basin from biodiversity, scientific perspective and socio-economical points of view. Due to the overfishing all sturgeon populations across the world were pushed to the brink of extinction, therefore the International Union for Conservation of Nature (IUCN) listed them as an endangered species group (Boscari et al., 2016), highlighting the need of special conservation programs. Out of six sturgeon species native to the Danube River Basin, only four can be found in our days: beluga sturgeon (*Huso huso*), stellate sturgeon (*Acipenser stellatus*), Russian sturgeon (*Acipenser gueldenstaedtii*) and sterlet (*Acipenser ruthenus*). Numerous studies regarding sturgeon life history have been conducted, questioning aspects such as fish stock management and assessment (Bacalbasa-Dobrovici, 1997, Bacalbasa-Dobrovici and Patriche, 1999, Banarescu, 1994, Hensel and Holcík, 1997, Navodaru et al., 1999, Paraschiv et al., 2006, Reinartz, 2002, Reinartz et al., 2012), reproduction (Jarić et al., 2010, Rosten et al., 2012, Vassilev, 2009, Vecsei et al., 2007), migration (Honț et al., 2015, Kynard et al., 2002, Paraschiv, 2011, Suciú et al., 2015, Suciú et al., 2013), hybridization (Birstein et al., 2005, Dudu et al., 2011, Ludwig et al., 2009) and diversity (Boscari et al., 2016, Dudu et al., 2008a, Dudu et al., 2008b, Ferguson et al., 2000, Holostenco et al., 2013, Holostenco, 2011, Onăra et al., 2014). Despite tremendous efforts to protect and conserve these species and the fact that they were and are an often-approached research topic, the present knowledge about their spawning and offspring behavior are incomplete, and some aspects are even completely unknown. Better understanding of spawning and offspring behavior will have a tremendous impact on long-term conservation strategies which can preserve and restore migratory sturgeon species from the Danube River Basin. To ensure a step forward in understanding sturgeon offspring ethology, we developed a pilot study regarding the 2016 sturgeon offspring diversity in a Lower Danube River (rkm 123) feeding area

community. This approach will allow us to draw a hypothesis about offspring behavior that can be applied in other feeding areas on the Danube River. This paper describes aspects of annual recruitment, fitness distribution and genetic diversity of two sturgeon species offspring.

## MATERIALS AND METHODS

### Capturing young of the year (YOY) and morphological measurements

Presence and abundance of two sturgeon species YOY (*Huso huso* and *Acipenser stellatus*) in the Lower Danube River (LDR) was monitored annually since year 2000, at river Km 123, on the Romanian side of the river downstream of Reni (Ukraine) (Suciu *et al.* 2004, Paraschiv & Suciu 2005). YOY sturgeons were captured using a 95 m long 20mm mesh size trammel net, drifting over 850 m of river bottom, where young sturgeons stop for feeding on benthic fauna, during their migration towards the sea. YOY captured were kept in a well aerated 20 l holding tank. Biometric measurements (total length - TL, standard length - SL, total weight - TW) were performed and recorded to the next millimeter and gram and digital photos of each fish including their code on the recording sheet were taken for the whole body as well as details for the shape and color pattern of their heads. All beluga sturgeons were tagged with Floy Fingerling Tags (FFT) by inserting of an elastic thread through the basis of their dorsal fin. Subsequently all YOY were released back in the river about 200 – 300 m downstream of their capture site.

### Sampling, DNA amplification and sequencing

Small fragments of the anal fin (~30 mg) were subjected to genomic DNA extraction using NucleoSpin® Food kit (Macherey-Nagel GmbH & Co. KG, DE) in accordance with manufacturer specifications. The total DNA was eluted in 50 µl Elution Buffer CE and then spectrophotometrically quantified. The PCR was carried out to partially amplify the mitochondrial D-loop region (~630 bp) using the Acip PHE 1F and Acip PHE 1R primers set previously described by Congiu *et al.* (2011). The sequencing was done using Dye-terminator method through a commercial service company (Macrogen Inc., NL). Sequencing results were assembled and edited in trace data format using DNA Dragon–DNA Sequence Contig Assembler Software (Hepperle - SequentiX - Digital DNA Processing). The D-loop sequences were aligned using the multiple-alignment ClustalW algorithm (Thompson *et al.*, 1994) implemented in MEGA 7 software (Kumar *et al.*, 2016).

### Genetic diversity and degree of admixture

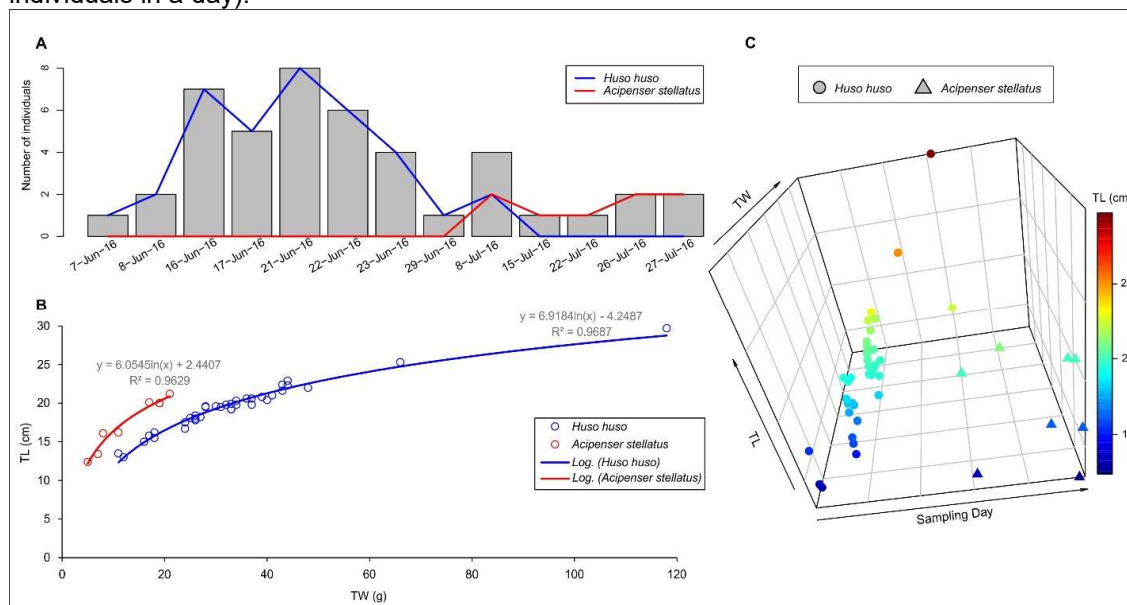
The genetic diversity of the sturgeon offspring from the Lower Danube River feeding area at rkm 123 was assessed using a data set comprising 44 D-loop sequences. For both sturgeon species, D-loop sequences were analyzed for haplotypes, genetic diversity ( $H_d$  - haplotype diversity,  $K$  – average number of differences,  $H_e$  - expected heterozygosity for polymorphic loci,  $\pi$  - nucleotide diversity) and selective neutrality (Tajima's selective neutrality test, Fu's neutrality test and Chakraborty's amalgamation test) with ARLEQUIN v.3.5 (Excoffier and Lischer, 2010). To illustrate the relationship between the haplotypes in both populations, SplitsTree software (Huson and Bryant, 2005) was used to construct statistical Median Joining networks for the D-loop sequences. Furthermore, the number of ancestral populations was estimated in R (R Core Team, 2016) using the LEA package (Frichot and François, 2015, Frichot *et al.*, 2014).

## RESULTS AND DISCUSSION

### Annual recruitment

The field sampling data show phased use of the feeding area by both species, with a relatively short period of overlap (Fig. 1A). The feeding ground seems to first be populated by beluga sturgeons offspring for at least one month, starting from the beginning of June until the beginning of July. The area was dynamically crowded over time, reaching the peak between 16 and 22 of June, followed by a progressive decrease until the end of June. At the beginning of July the number of beluga sturgeon offspring increased slightly, and at the same time the first stellate sturgeon offspring arrived in the feeding area. This feeding area crowding pattern seems to be a constant trait of beluga sturgeon YOY in this area, having also been observed in previous years (Paraschiv *et al.*, 2011, Paraschiv and Suciu, 2005, Paraschiv *et al.*, 2010, Paraschiv *et al.*, 2006). Paraschiv and Suciu (2005) identified three main arrival peaks using a Jolly-Seber model for open systems to describe their abundance dynamics. Moreover, the mark and recapture method proved that the offspring resided in the feeding area for at least 2 weeks. Two of the captured

individuals were released downstream (~300m) and then recaptured upstream in the sampling area, one of them after 14 days. Therefore, beluga sturgeon YOY exhibit strong gregarious behavior confirmed over time, since 2002 (Paraschiv and Suci, 2005), by multiple mark and recapture events (Onăra et al., 2014, Paraschiv, 2011, Paraschiv et al., 2011, Paraschiv and Suci, 2005, Paraschiv et al., 2010, Paraschiv et al., 2006). On the other hand, the stellate sturgeon offspring's shoal dynamic exhibits a strange pattern over time, having a maximum at arrival, followed by a slight decrease and then back to a maximum, but this time in a stationary stage until the end of the sampling period. Regarding body size parameters (TL, SL and TW), a common trend could be observed for both species. The individuals captured in the same day exhibit high variation in weight and even in length (Fig. 1C). The variation in weight was expected, due to differences in individual fitness and feeding success, but the high variation in length was not, since the individuals' ages are expected to be the same ( $\pm 1$  day). A possible explanation for the length variation is that the captured individuals are from different groups, originating from several spawning areas along the Danube River, that are spending different amounts of time feeding until they reach the sampling area. This hypothesis is supported by the smooth regression between body weight and total length for both species (Fig. 1B). The same TL~TW regression trend was previously reported by Suci et al. (2004), describing a power function distribution. The increased number of individuals from both species caught in the overlapping time and the body size parameters distribution reveals several behavioral traces, as follows. The beluga sturgeon offspring arrive in the feeding area in small and multiple groups, in stages, cohabiting together in the feeding area during this period and at the end they leave, also in stages. The overlapping, cohabitation time of the beluga and stellate sturgeon offspring represents a transitional period, when the beluga sturgeon offspring are leaving while the stellate sturgeon offspring are arriving gradually. Moreover, the stellate sturgeon offspring exhibit a more grouped and synchronized arrival (the captured number of individuals in the first day of occurrence is equal to the maximum number of captured individuals in a day).

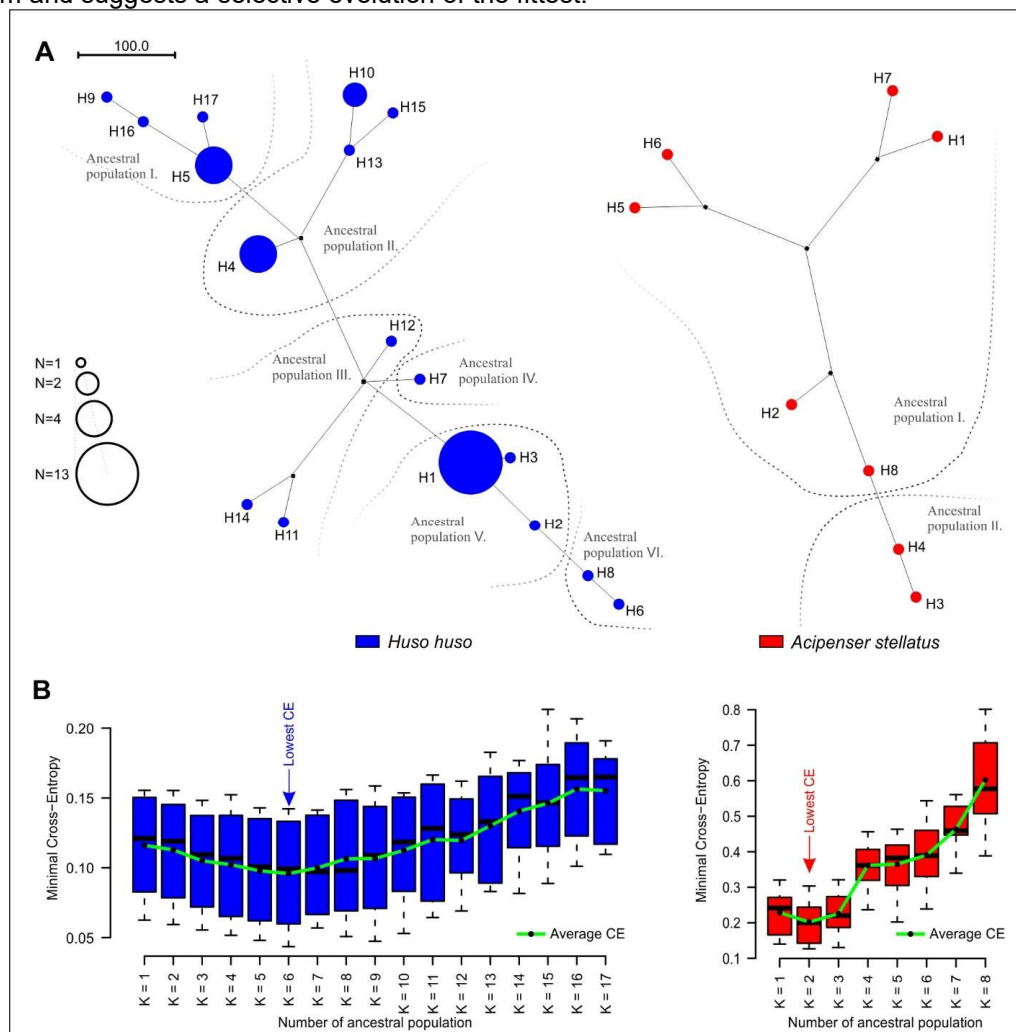


**Figure 1** The dynamic of daily catches in year 2016 and body size parameters of YOY sturgeons from the Lower Danube River feeding area at rkm 123. A – The time distribution of the total number of captured individuals (gray bars) and separately for each species (*Huso huso* – blue line, *Acipenser stellatus* – red line); B – The body weight and total length correlation degree (logarithmical regression and the associated function) for both species (*Huso huso* – in blue, *Acipenser stellatus* – in red); C – 3D plot of body size parameters variation per day.

#### Genetic diversity of the feeding area community

The genetic assay of the *Huso huso* offspring from the Lower Danube River feeding area at rkm 123 revealed 17 haplotypes (from 36 individuals), 4 of them being shared among individuals. Our data are in accordance with a previous genetic survey conducted using D-loop RFLP analysis that showed 4 haplotypes, all of them shared among 255 individuals sampled from the same area over 6 years (Onăra et al., 2014). The molecular diversity indices (Table 1) show a high level of genetic diversity mainly

determined by the high number of haplotypes with relatively large distances between them. The haplotypes network shows an evolution starting from multiple ancestral nuclei (Figure 3A) that explains the high level of genetic diversity. The selective neutrality and population equilibrium hypotheses (Ewens, 1972, Slatkin, 1996, Tajima, 1989, 1996, Watterson, 1975) were rejected through Tajima's D (Tajima, 1989) and Fu's FS (Fu, 1997) tests, suggesting a population expansion after a recent bottleneck event or most likely a significant degree of multiple populations admixture. The hypothesis of multiple populations admixture is strongly supported by Chakraborty's test (Chakraborty, 1990) of population amalgamation (observed number of haplotypes = 17, expected number of haplotypes = 18.58), suggesting that the higher number of expected haplotypes are due to an amalgamation of previously isolated populations. Previously, Onăra *et al.* (2014) identified, in different years of sampling, amalgamation events, highlighting as many as 3 distinct genetic groups in a single year. The genetic survey of the *Acipenser stellatus* offspring highlighted the presence of 8 haplotypes from a total of 8 individuals. The lack of haplotype fixation in the offspring population (the lack of sharing) could be caused by two equally likely reasons: (a) incomplete sampling – the sample size is too small to describe the entire offspring community (individuals with different genotypes were captured by chance, even though *de facto* these haplotypes are shared); or (b) the survival rate of the *Acipenser stellatus* offspring is so low that just a few individuals from the same parent pair are adaptable enough to survive. Moreover, the increased distance between haplotypes and the high nucleotide diversity are traces of a previous rapid demographic contraction. The Tajima's D and Fu's FS selective neutrality tests was not significant enough to reject the population trend towards equilibrium and suggests a selective evolution of the fittest.



**Figure 2** The haplotypes network and estimated number of ancestral populations among *Huso huso* and *Acipenser stellatus* species.

A – Median Joining networks for the D-loop sequences of the *Huso huso* (in blue) and *Acipenser stellatus* (in red) individuals. . The black ellipses indicate haplotypes not found in the specific population. The ellipses size is in accordance with the number of individuals. The haplotype distances are scaled in genetic distance, “weighted” Hamming distance (Bandelt et al., 1999). B – Estimated number of ancestral populations through minimal Cross-Entropy.

**Table 1** Genetic diversity among *Huso huso* and *Acipenser stellatus* species.

	N	N <sub>h</sub>	S	H <sub>d</sub>	K	H <sub>e</sub>	π	D	FS	Pr (K ≥ k obs)
<i>Huso huso</i>	36	17	150/723	0.8556	17.744	0.098	0.0204	-2.2435 ***	1.2314 ns	0.0124
<i>Acipenser stellatus</i>	8	8	108/734	1.0000	38.893	0.053	0.053	-0.7812 ns	0.0312 ns	-

Statistical significance: ns not significant; \* 0.01 < P < 0.05; \*\* 0.001 < P < 0.01; \*\*\* P < 0.001

N = Number of sequences; N<sub>h</sub> = Number of haplotypes; S = Number of polymorphic sites; H<sub>d</sub> = Haplotype diversity; K = Average number of differences; H<sub>e</sub> = Expected heterozygosity for polymorphic loci; π = Nucleotide diversity (average over loci); FS = Fu's FS test; Pr(K ≥ k obs) = Chakraborty's test.

Furthermore, to cross-validate the hypothesis of multiple populations' admixture in the feeding area, the minimal cross-entropy was checked to estimate the minimal involved evolutionary effort for the observed haplotypes. This analysis confirms the populations admixture, proving that beluga sturgeon offspring genotypes evolved from six ancestral population and the stellate sturgeon offspring from two (Figure 3B). Both body size parameters and genetic traits confirm the hypothesis of multiple populations' admixture in the feeding area. Therefore, sturgeon offspring feeding areas are in fact common nurseries used by these two sturgeon species. Also, we highlighted the gregarious behavior of the offspring of both species in the feeding area, most likely a survival strategy. The steep slope of the beluga sturgeon offspring's arrival in the feeding area suggests a specific behavior for each group, most probably dependent on their geographical origin. The unsynchronized arrival raises two possible hypotheses about beluga sturgeon behavior: (a) the females exhibit homing behavior (the offspring arrive in small groups with a slight phase shift, do to their inherited behavior) and (b) offspring behavior is driven by the most fitting survival strategy for each individual without any homing features (the timing issue is just a matter of individual fitness, food availability and geoclimatic background). On the other hand, stellate sturgeon offspring seem to have better timing and therefore a different survival strategy.

As these two sturgeon species have a critically endangered status (IUCN, 2010), the number of haplotypes observed in 2016 YOY highlights the reproductive success of a significant number of females. Therefore, the offspring of beluga sturgeon captured in 2016 originate from 17 genetically distinct females as was shown by mitochondrial genotyping. Similarly, the stellate sturgeon YOY captured in 2016 originate from 8 genetically different females.

The present paper presents a pilot study of sturgeon behavior that reveals several traits of offspring ethology, but is still just a tiny part of their entire behavior. Further analyses are needed to decode the cryptic behavior of sturgeon. Also, new experimental designs are required to try to answer questions such as: Do sturgeon species have homing behavior? What kind of pairing strategies do they exhibit? How was their behavior influenced or even shifted (geographically and temporally) by the construction of dams on the Danube River?

## CONCLUSIONS

Feeding grounds are an important class of habitats for the downstream migration of sturgeon offspring, representing common nurseries used in turn by the two sturgeon species analyzed in this paper. Our data, in accordance with previous surveys, together highlight a general tendency of crowding feeding areas, strong gregarious behavior and ethological peculiarities of cohabitation. Based on genetic data, we

confirmed the hypothesis of multiple populations' admixture in the feeding area. The convergence of the morphological and genetic data let us strongly assert that YOY of these two sturgeon species have distinct and complex downstream migration behavior. Overall, this study allowed us to set new hypotheses that should create a paradigm for future studies about the behavior of sturgeons in the Lower Danube River. In addition, the present paper presents, for the first time, evidence about the number of successfully reproducing females, as an indicator of recovery for these two sturgeon species in the LDR.

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