SOFIA UNIVERSITY "ST. KLIMENT OHRIDSKI",
FACULTY OF BIOLOGY,
DEPARTMENT OF GENERAL AND APPLIED HYDROBIOLOGY

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Borislava Kostadinova Margaritova

Study of the spawning and feeding habitats of the sturgeons in the Bulgarian section of the Danube River

SYNOPSIS
of a dissertation

For the awarding of an educational and scientific degree "Doctor"
in professional direction 4.3 Biological Sciences
Specialty “Hydrobiology"

Sofia, 2022

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PhD supervisors: Assoc. Prof. Dr. Eliza Uzunova,
Assoc. Prof. Dr. Lyubomir Kenderov

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The dissertation has a volume of 239 pages, including 21 tables, 19 figures and 65 appendices; 279 literary sources are cited, of which 35 are in Cyrillic and 244 are in foreign languages.

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The defense of the thesis will take place on January 10, 2023 at 1:00 PM in the Meeting Hall of the Faculty of Biology, Sofia University "St. Kliment Ohridski".

Scientific jury:

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List of abbreviations used in the text

MA	Ministry of Agriculture
MoEW	Ministry of Environment and Water
NBA	National Biodiversity Assessment
CPUE	Catch per unit effort
CWT	Coded Wire Tags
IUCN	International Union for Conservation of Nature
IRI	Index of relative importance
PIT tags	Passive integrated transponder
rkm	River kilometer
TL	Total length
WWF	World Wide Fund for Nature
YoY	Young of the Year

1 Introduction and literature review

The sturgeon species inhabiting the Danube River are included in the IUCN Red List and in the Red Book of Bulgaria. The factors responsible for the current status of this unique fish species are the direct result of human intervention in the life of the river. Today, after more than 10 years of a total ban on sturgeon fishing in the Danube River and the Black Sea, their status as an endangered species is still a fact. The reasons for the difficult recovery of sturgeon fish populations are linked to their specific biology – a long life cycle, late sexual maturity and specific requirements for breeding grounds.

The dissertation starts with a brief review of the biological characteristics of the sturgeons inhabiting the Danube River and the Black Sea, the state of the populations and the current measures for their conservation and restoration. From the review of the literature, the following conclusions were drawn about the current state and the gaps in knowledge about sturgeon fish and their habitats in the Bulgarian part of the Danube River:

- there is a lack of up-to-date data on sturgeons, which is why Bulgaria is reporting Sturgeon species under art. 17 of Habitats directive in the Data Deficiency (DD) category;
- the last available information on the spawning habitats of the *H. huso* in the Bulgarian section of the Danube is from 2002. There is no information available whether the other sturgeon species have adapted and spawn in places below the Iron Gate II Dam;
- the last research made in Bulgaria regarding the diet of sturgeons date from the 1950s and 1960s;
- it is unlcear whether the changes in the structure of benthic macroinvertebrate influence the food preferences of sturgeon species and – if they do – it is not clear how.

Updating information on the life of sturgeons will help to make complex decisions on case studies (hydropower projects, maintenance of shipping, etc.) that can be carried out in an environmentally friendly way.

But for this purpose, we should fill in those gaps in our knowledge about sturgeons so as to guarantee their conservation and restoration. Key factors in this regard are the sturgeon feeding and spawning habitats, therefore the efforts of the current dissertation work are focused on these two factors.

2 Objective and tasks

The aim of this dissertation is to determine the potential spawning and feeding habitats of sturgeons in the Bulgarian section of the Danube River with a view to their future protection and restoration.

To achieve this goal, the following tasks are set:

1) To determine the status of sturgeons in the Bulgarian section of the Danube River, by:

- Studying the species composition and sturgeon abundance;
- Determining the size structure and condition of sturgeons;

2) To revise the potential spawning habitats known in the past as sturgeon spawning grounds in the Lower Danube by:

- Studying sturgeon migration of different size-age groups;
- Researching the presence of sturgeon eggs and larvae in the area of potential and established spawning grounds in the past;
- Analysis of abiotic factors (hydromorphological, physical and hydrochemical) in potential spawning habitats;

3) To explore potential sturgeon feeding habitats through:

- Determination of sturgeon diet;
- Determination of quantitative and qualitative composition, autecological parameters of the macrozoobenthos in the sturgeon feeding habitats.

3 Materials and methods

3.1 *Study area and periods*

The studies were carried out in the Bulgarian territorial waters of the Danube River (between 847 and 375 river kilometers (rkm). The research on potential spawning habitats was carried out in the 2013 – 2019 period (April and May). The study covered the entire Bulgarian section of the Danube, as well as separate sections, determined on the basis of catches of young-of-the-year (YoY) and adult sturgeon, as well as the presence of certain abiotic factors (water level, temperature, water velocitiy, substrate and depth). For a total of 102 days 531 samples were collected with ichthyoplankton nets to examine sturgeon eggs, pre-larvae and larvae, so as to identify spawning habitats.

Sturgeon migration studies were conducted in the period from 2014 to 2021 (May – August). The samplings were conducted using bottom drifting trammel nets. The study was carried out in the Lower Danube River, mainly at river section between rkm 397 and 395 near Vetren village, and additional sections at rkm 815, rkm 576 and rkm 430. A total of 1,420 transects were surveyed for 231 days, of which 173 days in the Vetren region with 1,132 samplings. The transects were between 760 and 1980 m.

Sturgeon diet surveys were conducted in 2019 and 2021 in the Vetren area (at rkm 396).

During the research, the sturgeons caught included both wild specimens and sturgeons intially hatched in fish farms, which were tagged and released back into the river.

3.2 Methods

The sturgeon research method follows the fish monitoring methodology of the National Biodiversity Assessment (NBA) and, in particular, the "Approach to Sturgeon Monitoring in the Danube River" (Mihov, 2016), as well as the "Scientifically based program and methodology for field establishment of spawning sites of Danube sturgeons" (Ecological Center, 2014).

Sturgeons were tagged with external and internal tags with individual codes. Based on the information from the tagged sturgeon, the data on catch frequency, period during which the sturgeons remain in the study area, growth rate, survival rate, and downstream migration rate were analyzed.

Information was collected from fishermen on bycatch of sturgeon in the period 2018 – 2021.

The diet of sturgeon was obtained *in situ* using a gastric lavage method according to Haley (1998).

To assess food availability, macroinvertebrates from the bottom substrate were collected periodically from April to October, 2019 and 2021, and the study covered the entire Bulgarian section of the Danube. Bottom samples were collected using a hand-held net (500-μm mesh size) according to the multi-habitat method (EN 16150:2012) and a Van Veen dredger according to BDS EN ISO 10870:2012.

In the period September – October 2021, a hydrographic survey by sonar was carried out in order to investigate potential habitats of sturgeons. In total, 22 transects along the entire stretch of the Bulgarian section of the Danube River were investigated. During the survey, bottom samples were collected to identify the substrate and to validate the information obtained from the sonar on bottom hardness.

The following parameters were also investigated: water temperature (T, °C), dissolved oxygen ($mg.dm^{-3}$), pH, electrical conductivity (μs/cm), velocity ($m.s^{-1}$) and transparency of the water (m). Additional information on current velocity, depth and substrate, was provided by the published database from the Joint Danube Survey 3 hydromorphological study (Schwarz et al., 2014).

3.3 *Data Analysis*

Sturgeon abundance is represented as the catch per unit effort ($CPUE_{10ha}$). An area of 10 hectares has been chosen, because it is closest to the area usually covered with trammel nets. The relative growth rate relative to the mean weight gain of the fish (*RGR*, in $\%d^{-1}$) was calculated. Size structure and length–weight relationships (LWRs) of sturgeons were analyzed, including data from fish caught in the Danube River and by-catch in the Black Sea. The established allometric or isometric type of body length growth was tested by Student's t-test (Zar, 1984). Fulton's condition factor (*K*) and relative condition factor (*Krel*) were calculated separately for each species relative to the size class. In order to assess time and body-size-related variations, sturgeons were categorized by their body length. As sturgeons up to 35 cm TL belong to young-of-the-year fish, and specimens over 35 cm TL are classified as sub-adults and adults (Ivanov 1988; Rogin, 2011), two size classes were identified: L1 < 35.0 cm TL and L2 > 35.0 cm TL. The statistical significance level of the coefficient of determination (R^2) and 95% confidence limits of *a* and *b* were computed for all equations. All length–weight relations were statistically significant ($P < 0.001$). All analyses were performed using Statistical Package SPSS version 22 (SPSS Inc. Ltd.) and Excel software (Microsoft Office, 2016).

The rate of the accessibility of the fish to itsprey and the weight of the food components (wet mass, g) were calculated as a percentage of the total weight of the stomach contents. To assess the relative importance of the different prey in the sturgeon diet, the frequency of occurrence (*Fi*) and the relative abundance (*Ai*) were calculated for each taxon, as well as the index of relative importance (%*IRIi*) of the different preys (Hynes 1950, Manko 2016).

The graphical method of Costello (1990) was used to illustrate feeding strategies and prey importance as a function of prey-specific abundance and frequency of occurrence. Feeding strategies (generalisation or specialisation) were analysed by plotting *Fi* and *Ai*.

Diet overlap between sturgeon species was calculated by the Schoener index (*Is*) (Schoener 1970).

The density of each macrozoobenthic taxon (Di, ind.m^{-2}) from the bottom substrate was calculated based on the relative abundance of each taxon in the sample and the number of sample sites. Published information (database in the program Asterics 4.04 and at www.freshwaterecology.info) on autecological parameters of benthic macroinvertebrates found in stomach contents was used to categorize sturgeon feeding habitats.

4 Results

4.1 Species composition, abundance, size and catch periods

A total of 952 sturgeons were caught in the period 2014 – 2021. Of these, 864 were young-of-the-year (YoY) (TL < 35 cm, L1) and 88 were adults (TL > 35 cm, L2). Eighty-seven sturgeons were caught more than once. A total of 31 YoY sturgeon were specimens from restocking.

The following **adult** sturgeons were caught: 76 specimens of *A. ruthenus*, 7 *A. stellatus*; 1 *H. huso* and 1 *A. gueldenstaedtii*. Three hybrids (*A. ruthenus* × *A. stellatus* and *A. ruthenus* × *A. gueldenstaedtii*) were caught. The largest number of sturgeon specimens was caught in 2019, the highest relative abundance of adult sturgeon was recorded in 2021. For the 8-year study period, the average relative abundance of sturgeons was 0.063 ind./net/ha.

Out of a total of 864 **YoY** catches belonging to four sturgeon species - *A. ruthenus*, *A. stellatus*, *A. gueldenstaedtii* and *H. huso*, 713 were caught in the Vetren region (396 rkm) in the period 2014 – 2021. Species *A. ruthenus* dominate with 599 specimens, followed by *A. stellatus* – 99 specimens, *H. huso* – 7, and one specimen of *A. gueldenstaedtii*. Seven YoY hybrids (*A. ruthenus* × *A. stellatus*) were caught.

The species *A. ruthenus* was recorded in 6 out of a total of 8 years, followed by *H. huso* captured in 3 years, and YoY hybrids *A. ruthenus* × *A. stellatus* recorded in 4 years during the period 2014 – 2021. The species *A. stellatus* was captured only in 2 years, *A. gueldenstaedtii* only in 1 year (Figure 4-1).

The average relative abundance of YoY specimens over a period of 8 years was 0.289 ind./net/ha. The highest number of YoY specimens and the highest relative abundance were recorded in 2018, followed by 2019 and 2017. In other years, single specimens were registered.

Only seven YoY specimens *H. huso* were captured during the 8-year study period. All YoY *H. huso* specimens caught at the Vetren site were quite uniform in length and weight: 219 – 297 mm total length (TL, mm) and an average weight (W, g) of 68.86 g. With a total effort of 1,132 nets, the $CPUE_{10ha}$ for the entire study period is 0.003. Species *H. huso* was registered in 2014, 2017, 2019, the most successful being 2017 with five specimens, while during the other years of the study only one specimen per year was caught (Figure 4-1).

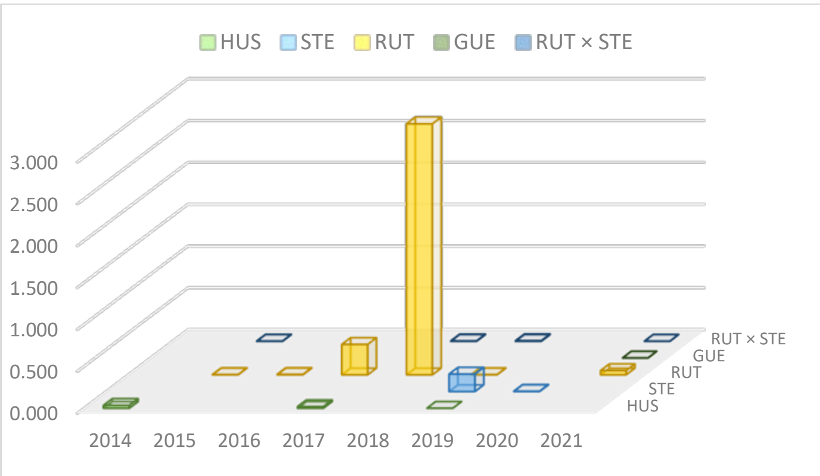

Figure 4-1. Relative abundance of young-of-the-year sturgeons throughout the years of the study conducted from 2014 to 2021 (GUE – *A. gueldenstaedtii*, HUS – *H. huso*, STE – *A. stellatus*, RUT – *A. ruthenus*, RUT × STE – *A. ruthenus* × *A. stellatus* hybrid).

YoY specimens of *A. ruthenus* had 148 – 290 mm TL and average weight of 44.04 g. The $CPUE_{10ha}$ for the entire study period was 0.290. In 2014 and 2020, the species was not registered, while in other years it was registered with different abundance (Figure 4-1). The largest number of specimens was registered in 2018, followed by 2017. In the other years, single specimens were caught.

YoY specimens of *A. stellatus* had 100 – 300 mm TL and a weight between 5 and 74 g. The $CPUE_{10ha}$ for the whole study period was 0.048 (Figure 4-1). During the 2014 – 2018 period and in 2021 *A. stellatus* was not registered. The main catches of the species occurred in 2019 with 97 specimens ($CPUE_{10ha}$ = 0.210), while in 2020 only two specimens were caught ($CPUE_{10ha}$ = 0.005).

For the entire period of the study, only one specimen of the *A. gueldenstaedtii* was caught (July 2021), with a total length of 176 mm (Figure 4-1).

Seven YoY *A. ruthenus* × *A. stellatus* hybrids or 0.98% of the total sturgeon catches were recorded for the entire study period. Specimens of *A. ruthenus* × *A. stellatus* had a total length of 182 – 278 mm and a weight between 19 and 73 g. The $CPUE_{10ha}$ for the study period was 0.003 (Figure 4-1).

Outside the area of the village of Vetren, only 9 YoY sturgeons (7 *A. ruthenus*, 2 *A. ruthenus* × *A. stellatus* hybrids) were caught, in the area of Tutrakan (440 rkm) and Marten (477 rkm).

Of the 87 recaptured sturgeons, 2 specimens were caught in the Black Sea, 1 in the section of the Danube River in the Belene (576 rkm) and the rest - in the Vetren region (396 rkm). For the period 2015 – 2021, no recaptures were reported in 2016 and 2020. Although only one sturgeon specimen was recaptured in 2015, it constituted 50% of the total catch for the year.

The most frequently recaptured species were *A. ruthenus* (59 specimens), followed by *A. stellatus* (n =24), *H. huso* (n = 2), *A.*

gueldenstaedti and hybrid *A. ruthenus* × *A. stellatus* with one specimen each.

The duration the sturgeons spent in the studied section of the Vetren region varied from 2 to 9 days (average 3.8), some specimens were caught between 2 and 7 times (average 2.5).

<u>Sturgeon by-catch</u>

For the period from 2018 to 2021, according to data from fishermen on bycatches, a total of 130 specimens of 4 sturgeon species were registered. Twenty-five of the fish were caught as bycatch in the Danube River, and another 105 in the Black Sea. The highest number of sturgeons was reported in 2019 (63 specimens), followed by 2018 (32 specimens).

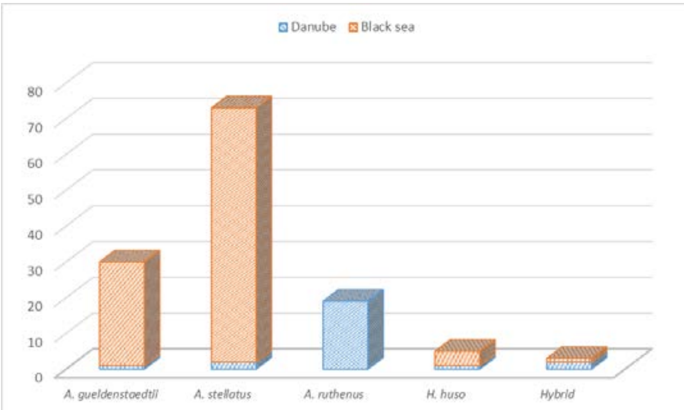

Figure 4-2. Registered sturgeons as by-catch in the Bulgarian sections of the Danube River and the Black Sea for the period 2018 – 2021 (according to data from fishermen).

The most frequently registered by-catch species is the *A. stellatus* (73 specimens), followed by the *A. ruthenus* with 19 specimens (Danube). Of the 30 registered specimens of *A. gueldenstaedti*, 19 are restocked individuals and 11 are wild fish. A total of 5 specimens of the species *H. huso* were registered (1 YoY from stocking in the Danube, 4 adults in the Black Sea). In total, 3 adult hybrid sturgeon fish were registered (2 from the Danube River and 1 from the Black Sea) (Figure 4-2).

Length-weight relationships and relative condition factors of sturgeon species

Length-weight relationships (LWRs), Fulton (*K*) and relative condition factors (*Krel*) for *A. ruthenus*, *A. gueldenstaedtii* and *A. stellatus* from the Danube River and the Black Sea were researched. A total of 790 specimens were measured and analyzed. More than 80% of the specimens were YoY fish with total length (TL) up to 35 cm. The values of parameter *b* for YoY and juvenile specimens with length up to 35 cm ranged from 2.433 (*A. ruthenus*) to 2.859 (*A. gueldenstaedtii*). For specimens with TL > 35 cm the value of parameter *b* was 3.227 (*A. stellatus*) and 3.668 (*A. ruthenus*). For *A. ruthenus* and *A. stellatus* specimens with length up to 35 cm, growth type was negative allometric, while at *A. gueldenstaedtii* was isometric. Larger specimens of *A. ruthenus* (> 35 cm TL) and *A. stellatus* (*b* = 3.227) exhibited positive allometric growth. Relative condition factor (*Krel*) ranged from 1.003 (*A. gueldenstaedtii*) to 1.144 (*A. ruthenus*).

The relative growth rate of *A. ruthenus* grown in fish farms and restocked in the Danube River was 1.98% d^{-1}, while for wild *A. ruthenus* it was between 1.22%d^{-1} (in 2017), 1.91%d^{-1} (2018) and 2.85%d^{-1} (2021). Growth rate for the species *A. stellatus* was 1.50%d^{-1} (average 2.05 g and 1.05 mm per day). The relative growth rate of restocked *A. gueldenstaedtii* was 1.53% d^{-1} (2.6 g/day).

4.2 *Potential spawning habitats of sturgeons*

A study of sturgeon migration

The migration of adult sturgeons to the spawning grounds (upstream) was tracked by fish tagged with acoustic transmitters: two *A. stellatus*, one *H. huso* and one *A. ruthenus.* Signals from the marked specimens were detected only by a mobile acoustic receiver within the day of tagging, but there is no data on a detected signal from the transmitters of the stationary receivers located on the Bulgarian bank of the Danube. CWT-tagged specimens of *A. gueldenstaedtii* from the restocking in

Belene region (2019) were recaptured in the Danube Delta and in the northernmost regions of the Bulgarian Black Sea coast. The estimated average speed of their downstream migration is between 18.6 and 5.6 km/day. For restocked and tagged YoY *H. huso* (2020) in the Belene region and caught in the Bulgarian and Romanian sections of the Danube River, a downstream movement speed of between 30 and 45 km/day was calculated. The downstream movement rate of newly hatched wild *A. ruthenus* was 3.91 km/day, calculated based on the distance from a station at rkm 576 (Belene) to station at rkm 396 (Vetren).

Abiotic factors in potential spawning habitats

The period, in which the water temperature is at the optimal values for sturgeon reproduction and at the same time the water reaches a spring peak (s) of the water level / discharge, occurs annually in the period March – April. The estimated breeding period for *H. huso* and *A. ruthenus* is in the period March 9 – April 21, with an average water temperature of 7.8°C (range 5.8 – 9.1°C).

The water depth in the studied potential sturgeon spawning habitats is over 2 – 5 m. In the Novo selo (839 – 835 rkm), Tsibar (717 – 714 rkm), Vadin (651 – 647 rkm), Nikopol (595 – 594 rkm), Belene (578 – 573 rkm), Mechka (516 – 512 rkm), Dolno Ryahovo (420 – 415 rkm) and Vetren (399 – 393 rkm) areas, sections deeper than 10 m have been established.

The hardness of the bottom (H) in the areas of the studied potential spawning habitats according to the data generated by the sonar indicates places with the highest values in the Baikal (643 – 640 rkm), Lom (746 – 745 rkm), Florentin (829 – 827 rkm) and Novo selo (839 – 835 rkm) regions (H = 0.5). The bottom substrate in the studied sections is mainly composed of: round stones and rocks in the section in the Gomotartsi (817 – 814 rkm) and Novo selo (839 – 835 rkm) areas; stones and gravel near Nikopol area (595 – 594 rkm); gravel and coarse sand in the Belene area (578 – 573 rkm); gravel in Lom area (746 – 745 rkm); gravel in the Vetren area (399 – 393 rkm).

Localization of sturgeon spawning grounds

No sturgeon eggs and larvae were detected using ichthyoplankton nets during the seven-year survey period.

In order to determine the spawning sites on the basis of the young-of-the-year sturgeon catches, back-calculations were made using the estimated date of breeding, the length of the specimens and their downstream movement speed.

4.3 Sturgeon diet and feeding habitats

In 2019 and 2021, the diet of 78 sturgeons from the species *A. gueldenstaedtii*, *A. stellatus, A. ruthenus* and the hybrid (*A. ruthenus* × *A. stellatus*) was studied. Specimens were caught in the period June – August, mainly at rkm 396. Single samples were collected at 574 and 440 rkm.

The organisms found in the stomach contents of sturgeons belong to six main groups: insects (Diptera: Chironomidae, Simuliidae and Trichoptera), crustaceans (Amphipoda, Mysida, Isopoda), molluscs (Bivalvia, Gastropoda), oligochaetes (Oligochaeta), fish (Gobiidae) and parasites (Nematoda, Acanthocephala). The presence of detritus in various volumes was recorded in the stomachs.

A comparison of sturgeon diet data across the two study years revealed that the amphipod *O. obesus* was the dominant prey for *A. gueldenstaedtii* (index of relative importance, %IRI = 97.75), *A. stellatus* (%IRI = 94.41) and *A. ruthenus* × *A. stellatus* (%IRI = 87.8%), all of which were specimens below 35cm TL (L1). For the species *A. ruthenus*, the index of relative importance for *O. obesus* is minimal (0.28% for L1 and 1.12% – L2). Species of the genus *Chelicorophium* are the main prey of the YoY specimens (L1) of the *A. ruthenus* with the index of relative importance of 75.36%. In the hybrid A. *ruthenus* × *A. stellatus* relative importance of *Chelicorophium* spp. is also relatively high (%IRI = 10.90), while for *A. ruthenus* of size class L2 and species *A. gueldenstaedtii* and *A. stellatus* of L1 it is insignificant (IRI between

0.01% and 0.06%). Chironomidae larvae were a component of the diet in all species and size classes with relative importance ranging from 20.93% in *A. ruthenus* from L1 to 1.07% in *A. ruthenus* from L2. The mussel *Corbicula fluminea* is the main food item for the adult *A. ruthenus* (%IRI = 97.74), while it was not recorded for the other sturgeons, with the exception of 1 specimen in *A. ruthenus* from L1. Species of the genus *Hydropsyche* were found in *A. ruthenus* (in L1, %IRI = 3.29) and in the hybrid specimens *A. ruthenus* × *A. stellatus* (%IRI = 0.08). The rest of the food items found in the stomach contents of the sturgeon included in the "Other" group are of very low relative importance (between 0.003 and 0.13%) (Figure 4-3).

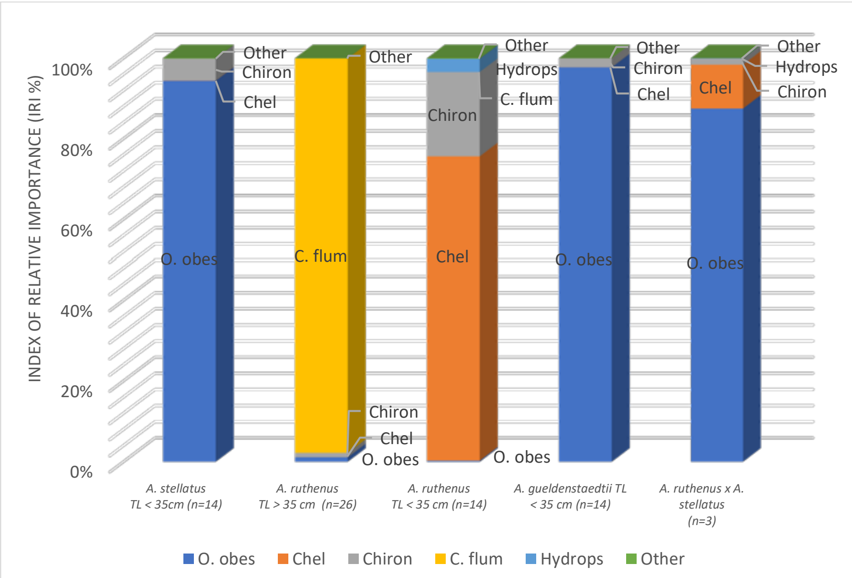

Figure 4-3. Index of relative importance (IRI %) of different prey items consumed by sturgeon species collected in the Lower Danube River (2019 and 2021). (L1 – specimens with total length TL < 35 cm, L2 – specimens with TL > 35 cm, Chel – *Chelicorophium* spp., Chiron – Chironomidae, C. flum – *C. fluminea*, O. obes – *O. obesus*, Hydrops – *Hydropsyche* spp., Other – all items with IRI less than 1.00 %)

In terms of feeding strategy, no specializations were observed in sturgeons, but generalization in relation to Chironomidae larvae was observed in the L1 species *A. gueldenstaedtii*, *A. stellatus* and *A. ruthenus* (Figure 4-4).

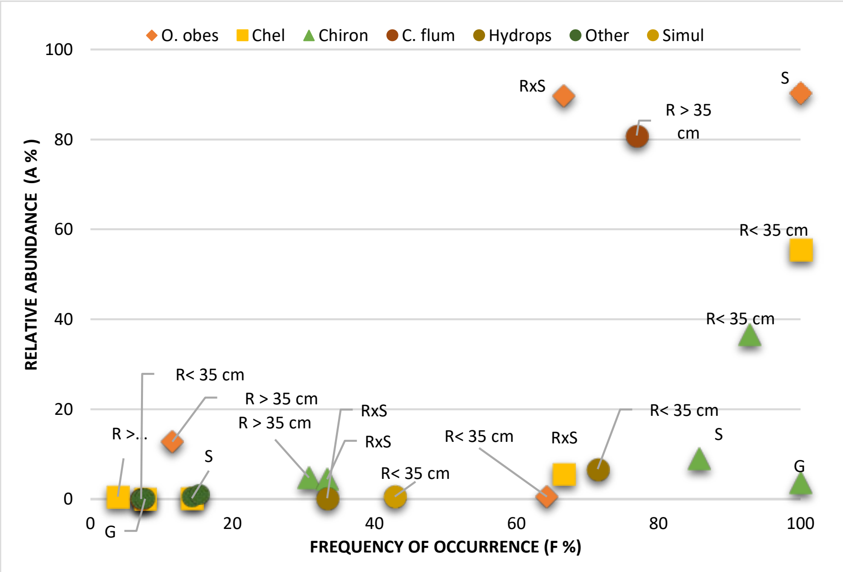

Figure 4-2. Feeding strategy of sturgeon species (R – *A. ruthenus*, S – *A. stellatus*, G – *A. gueldenstaedtii*, R×S – *A. ruthenus* × *A. stellatus*, Chel – *Chelicorophium* spp., Chiron – Chironomidae, C. flum – *C. fluminea*, Hydrops – *Hydropsyche* spp., O. obes – *O. obesus*, Simul – *S. colombaschense*, Other – all items in which the abundance was less than 1.00%).

When comparing the most important food items in sturgeon stomach contents, an almost complete diet overlap was detected between *A. gueldenstaedtii*, *A. stellatus* and the hybrid *A. ruthenus* × *A. stellatus* with values of the Schoener index above 0.84. This overlap was observed when comparing not only the individual species, but also different months. Meanwhile, no overlap was noticed between the diet of *A. ruthenus* and the other three species.

The macrozoobenthos as a trophic base for sturgeons

The diversity of invertebrates found in the bottom substrate samples of the studied river sections includes 13 groups (Turbellaria, Gastropoda, Bivalvia, Polychaeta, Oligochaeta, Hirudinea, Crustacea, Ephemeroptera, Odonata, Heteroptera, Trichoptera, Coleoptera and Diptera) and 65 taxa. The largest number of species is observed in the representatives of the Gastropoda with 15 taxa, while the Crustacea with 472 ind.m^{-2} have the highest number. The most abundant was the mussel *C. fluminea* with 256 ind.m^{-2}, followed by *Dreissena polymorpha* with 123 ind.m^{-2} and *Dikerogammarus villosus* with 117 ind.m^{-2}.

5 Discussion

5.1 *Status of sturgeon populations in the Danube River*

Direct comparison of the results from the monitoring carried out in the Lower Danube River is difficult due to the fact that there is no unified method of calculating the CPUE. Due to the fact that different countries use different net and transect lengths, data needs to be recalculated to allow for comparison. During the sturgeon monitoring carried out by Romanian scientists at rkm 118, the studied area of the river bottom was about 8 ha (Suciu 2008), whereas during our research the area covered by the nets was about 10 ha, which leads to the need for data recalculation. According to our data, the calculated $CPUE_{10ha}$ of the species *H. huso* for the entire study period is 0.003, which is very low when compared with the recalculated $CPUE_{10ha}$ results from the monitoring at rkm 118 for the 2000 – 2008 period (Suciu 2008) when it was 2.186. Direct comparison of the results from the monitoring at rkm 118 and monitoring done by us at rkm 396 is also not possible, not only due to the time difference in the monitoring but moreover because of the great distance between the two monitoring stations.

With only one registered individual of the species *A. gueldenstaedtii* in more than 1,100 nets, the abundance is close to zero. The recalculated 0.116 $CPUE_{10ha}$ for the years 2000 – 2008 as a result of the YoY monitoring at rkm 118 (Suciu 2008) is quite high, but for the last 10 years no YoY *A. gueldenstaedtii* have been registered even at the Romanian monitoring site (Iani et al. 2019).

These results indicate a continuing downward trend in the recovery of sturgeon populations in the Lower Danube. This negative trend indicates the long-term adverse effect that factors such as overfishing, disruption of river connectivity, etc. have on populations. Obviously, the ten-year total ban on fishing, the stocking of small sturgeon fish, the improvement of water quality reported after the period of the 1990s cannot compensate for the drastically reduced numbers of these fish. It can be concluded that namely the intensive and irrational fishing in the past and the interruption of migration are the main reasons for the current state of sturgeon populations (Navodaru et al. 1999) Various anthropogenic factors have a significant impact on the abundance of sturgeon populations in the Danube River (Lenhartdt et al. 2006, Jarić et al. 2010, Gough et al. 2012).

In recent years, there has been an increasing presence of alien sturgeon species in the Danube, as a result of intentional and/or unintentional release of specimens from fish farms (Lenhard et al. 2008, Ludwig et al. 2009). A reason for the increased proportion of sturgeon hybrids is the limitation of the size of suitable spawning sites, which leads to the gathering of different sturgeon species in the same habitats and interbreeding between them (Holčik 1989, Lenhardt et al. 2008). Some of the hybrids entered the Black Sea (Tzekov et al. 2008), and others remained in the river. In the course of the present research, we found one- or two-year old hybrids (*A. ruthenus* × *A. stellatus* and *A. ruthenus* × *A. gueldenstaedtii*) in the Danube, we also obtained similar by-catch data from fishermen at the Black Sea, which confirm the natural hybridization between sturgeon species. Another reason for the presence of hybrids in the Danube can be found in the restocking of sturgeon in the river.

Growth rate, length-weight relationships and condition factors are indicators of the health of fish populations (Martin-Smith 1996, Froese 2006). Ceapa et al. (2002) studied *A. stellatus* in the Romanian section of the Danube River and found negative allometric growth (for males, n = 128) in adults, whereas we reported this type of growth in juveniles of the species. For adult specimens of *A. stellatus* (> 35 cm TL), positive allometric growth was observed. Fazli & Moghim (2014) determined a positive allometric growth pattern for *A. gueldenstaedtii* from the Caspian Sea, while Mousavi & Ghafor (2014) indicated isometric growth for the same species, which was also found in our studies of YoY *A. gueldenstaedtii* from the Bulgarian section of the Danube. Our results provide an appropriate estimate of the length-weight relationships when the parameter *b* is between the expected ranges of 2.5 – 3.5 indicated by Froese (2006). Only in adult *A. ruthenus* the parameter *b* shows a higher value ($b = 3.688$, $R^2 = 0.936$), but similar results were also presented by studies of *A. ruthenus* in Russia (Podlesny 1958, Belyaeva et al. 1989). The results obtained for negative allometric growth inYoY *A. ruthenus* differed from the observed isometric growth ($b = 2.938$) in age 0+ and 1+ individuals by Lenhardt et al. (2004) in a morphological analysis of the population of *A. ruthenus* from the Serbian section of the Danube.

In our results for *A. stellatus* the Fulton’s condition factor has the lowest value (0.300), while *A. gueldenstaedtii* has a higher value (0.514). Similar values for the Fulton’s condition factor were reported by Mousavi & Ghafor (2014) with 0.352 for *A. stellatus* and 0.563 for *A. gueldenstaedtii*, respectively, with similar values also observed for the relative condition factor.

The studied YoY naturally hatched sturgeon in the river showed a good growth rate. Part of the released YoY sturgeons in the period 2014 – 2020 were also registered in our catches, with higher values for the relative growth rate compared to the relative average fish weight gain –

1.53%d^{-1} for *A. gueldenstaedtii* and 1.98 %d^{-1} for *A. ruthenus*, as well as higher values for the condition factor, which may be due to the fact that specimens were hatched in a farm where they were fed *ad libitum* for the first three months. Despite conflicting opinions on the effectiveness and benefits of sturgeon stocking in the Danube (Vassilev 2005, Ludwig et al. 2009, Apostolou et al. 2016), our data show that restocked sturgeons have a good growth rate, and the specimens successfully migrate and adapt well to the conditions in the river and in the Black Sea.

5.2 *Sturgeon spawning habitats*

It is known that until 1972, the spawning grounds of the species of *H. huso*, *A. stellatus* and *A. gueldenstaedtii* in the Danube River were located mainly above 942 rkm, and during the breeding migration they even reached 2,380 rkm (Hensel & Holcík, 1997). After the construction of the Iron Gate I hydroelectric power plant, which is a physical barrier to the movement of fish, the breeding migration of sturgeons upstream in the Danube is practically interrupted (Hensel & Holcík 1997, Suciu & Paraschiv 2016). At present, there is direct and indirect evidence that sturgeons of different species, as well as of the same species, adopt different tactics in solving this vital issue of their survival. Evidence for the choice of the first possibility is the our observations of the behavior of marked specimens of sturgeon show that when they reach the area in front of Iron Gate II (863 rkm), after several days of unsuccessful attempts to pass further up, they return back to the Black Sea, probably without accomplishing reproduction (Kynard et al. 2002, Hont et al. 2018). The other option for the sturgeons is to breed in the area currently accessible to them up to 863 rkm. However, for the realization of this scenario, two important conditions must be met, which can be defined as key in terms of successful natural reproduction of sturgeons in the Lower Danube. The first condition is related to the availability of suitable spawning habitats in the area of the river below Iron Gate II (Hont et al. 2018, Suciu et al. 2018). The second condition is related to the question of whether the sturgeons will return to the places where they themselves started their life (homing behavior), or they will look for new, alternative spawning habitats. The fact that in the last 8 years we caught more than 850 wild sturgeon specimens in the river under Iron Gate II (only in the Bulgarian sectoin), of which 746 were YoY, could be taken as indirect evidence of sturgeon reproduction in the Lower Danube. For one of the currently most numerous sturgeon species, *A. ruthenus*, we can make the definite conclusion that it reproduces in the Lower Danube and the fragmentation of the river at Iron Gate II (863 rkm) does not have such a significant impact on its natural reproduction. Proof of this is the fact that in the catch of sturgeon, *A. ruthenus* dominate both among the YoY specimens and among the adult fish.

In addition to YoY *A. ruthenus*, our catches also include YoY *H. huso*, *A. stellatus* and *A. gueldenstaedtii*, although in quantities ten times smaller than *A. ruthenus*. If we accept the presence of these fish as proof of the reproduction of sturgeon species in the Lower Danube, it is extremely important to identify the potential habitats where this is possible in the Bulgarian section, in order to take all possible measures to protect these places. Identification of spawning grounds can be done directly by capturing eggs and newly hatched larvae. After 2003, the only confirmed direct spawning site in the Lower Danube is at 118 rkm (Suciu et al. 2021). Despite our nearly ten-year-long effort to search for eggs and newly hatched sturgeon larvae, the discovery of which would direct us to the exact localization of the spawning grounds, their location was identified indirectly – based on back-calculations. The main variables in the model for identifying the location of sturgeon spawning habitats in the Lower Danube include water temperature, water level (to estimated date of breeding), the length of the specimens and their downstream movement speed.

According to Suciu et al. (2005), spawning of the *H. huso* and *A. ruthenus* in the Lower Danube River occurs the day after the spring peak (s) at the water level / discharge, at water temperatures above 6°C. This relationship between the two factors allows us to predict the specific days in a given year when we should expect breeding of *H. huso* and *A. ruthenus*.

Since both the duration of embryonic development and the behavior of larvae after hatching are known, it is possible to reconstruct the likely path taken by larvae and juvenile sturgeons downstream from hatching sites. It is also possible to follow this path in time, i.e., to predict on which date in which section (river kilometer) the fish will be found. On the other hand, these predictive models can be compromised by the influence of various anthropogenic factors mostly related to the modification of the natural hydrological regime of the river (Veshchev 1994). The operational mode of Iron Gate II Dam can cause a sharp increase in flow and water velocity, which will lead to a forced acceleration of the process of movement of larvae and small sturgeons downstream, or at least of those that have not found shelter in slower sections of the river. This was observed in 2020 when the speed of movement of three-month-old *H. huso* downstream reached 30 – 45 km/day as a result of the sudden rise in the water level by 3.5 m in just 15 days as a consequence of the release of water from the Iron Gate II.

The arrival of YoY *H. huso* at rkm 396 between 85 and 100 days after the calculated date of post larvae downstream movement suggests the spawning sites remained the same for the whole period of the monitoring, or are at least located in the same river section. Based on the data on the arrival of *H. huso* and *A. ruthenus* YoY from the known spawning site to the monitoring site in the Romanian section of the Danube (Onără et al. 2011), it was calculated that the speed of downstream migration is 4.18 km/day. A very similar speed – 3.91 km/day of downstream migration of *A. ruthenus* was calculated based on our data of finding a 106 mm YoY *A. ruthenus* on 8 of June 2015 at rkm 576 near the town of Belene, and the first arrival of *A. ruthenus* on 24 of July at rkm 396 near Vetren. Based on this calculated speed of about 4 km/day, it could be determined that the spawning sites are located about 340 – 440 kilometres upstream of rkm 396, i.e. between rkm 750 – 800 (town of Lom to town of Vidin), which fits quite accurately the location of spawning sites of *H. huso* between rkm 755 – 840 given by Vassilev (2003).

Young-of-the-year *A. ruthenus* arrived at Vetren station in about 73 – 88 days after the calculated date of post larvae downstream movement indicating possible spawning grounds between rkm 700 – 763 (Kozloduy – Archar) (figure. 5-1.). But because we are back-calculating the time of the first arrivals and not the last ones, it is possible that the spawning grounds are stretched even further upstream - between rkm 763 and Iron Gates II.

It is still not known if the spawning of *A. stellatus* is somehow dependent on the Danube water level, thus the potential spawning date of *A. stellatus* can not be calculated precisely. However, based on the fact that the mean length of YoY *A. stellatus* almost matches the mean length of the YoY *A. ruthenus* at Vetren station, it could be concluded that the spawning sites of *A. stellatus* are in the same river section as those of *A. ruthenus* – between rkm 700 and 763. The registered high frequency of natural *A. ruthenus* × *A. stellatus* hybrids is another confirmation of this assumption. According to Suciu (2008) the free embryos of Danubian populations of *A. stellatus* are pelagic for 11 – 12 days and are transported downstream by the currents at a velocity of up to 40 km/day. Our data do not support such behaviour of free embryos. Simple calculation shows that if the free embryos drift passively with the currents for 12 days at a speed of 40 km/day, then they will be transported 480 km downstream for the given period. This would suggest that *A. stellatus* should arrive at Vetren at the stage of post larvae just 10 days after hatching, considering that Vetren station is located at rkm 396 and Iron Gates Dam II, which sturgeons cannot pass through, is at rkm 863. This however is not supported by collected data. Instead, *A. stellatus* are registered at the Vetren station at the age of about 3 to 4 months old and at a total length very similar to that of *A. ruthenus* and *H. huso*, which suggests that at least part of the free embryos of *A. stellatus* share similar patterns of downstream migration with *H. huso* and *A. ruthenus* and are not passively transported downstream over such long distances during their early stages of development.

There were two specimens of *A. stellatus* measuring 100 mm and 130 mm in length respectively, arriving at Vetren station about a month earlier than the main cohort of *A. stellatus*. According to Ivanov (1988) at this length they should be about 35 – 50 days old, which means their spawning grounds would be located between rkm 535 – 600 (Vardim – Belene – Nikopol) (figure 5 1.). The catch of a female *A. stellatus* at rkm 576 (Belene) in May 2017 with a swollen urogenital area confirms this river section as a spawning ground for the *A. stellatus*.

Back-calculation based on the length of the specimen *A. gueldenstaedtii* and the fact that it arrived at Vetren station together with the *A. ruthenus* and measures a similar length, leads to the plausible assumption that the spawning grounds are close to the ones for *A. ruthenus*, probably between rkm 680 – 700 (Oryahovo – Kozloduy) (figure 5-1.). This first registration of YoY of *A. gueldenstaedtii* in the Danube, after a long period of absence, comes 3 years after the fist registration of restocked *A. gueldenstaedtii* returning to the Danube for spawning (Iani et al. 2019). According to Iani et al. (2019) any YoY recruitment observed since 2010, should be the result of an active spawning event of the stocked *A. gueldenstaedtii* individuals.

The hybrids *A. ruthenus* × *A. stellatus* usually arrive at the Vetren station together with YoY *A. ruthenus* and measure a similar length. The high frequency of these natural hybrids suggests that the two species share the same spawning grounds and a similar spawning behaviour. The hybrids seem to be healthy and viable, and have similar length and weight to the *A. ruthenus* they arrive with.

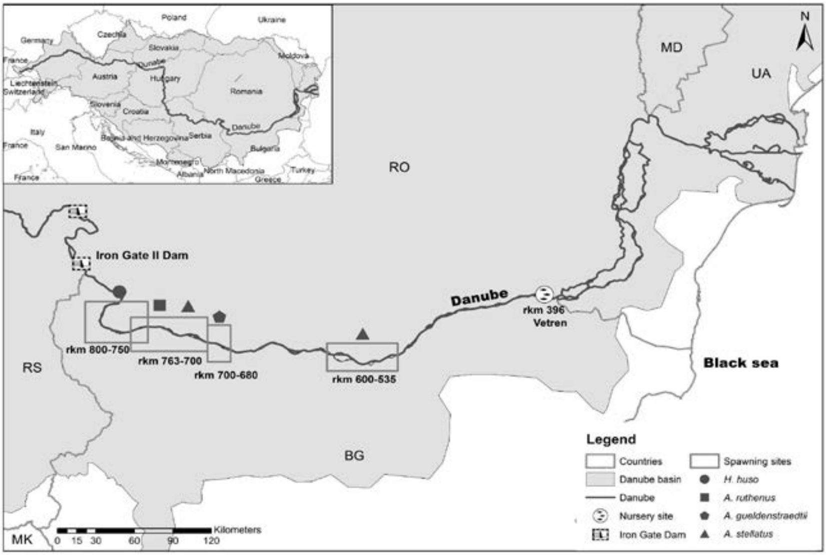

Figure 5-1. Map of the Lower Danube River, indicating the monitoring station (nursery site) at Vetren rkm 396 and the calculated potential spawning sites along the Bulgarian section of the river.

Key characteristics of the spawning habitats are the bottom substrate and the current velocity. According to the published database from the Joint Danube Survey 3 (JDS 3) hydromorphological study (Schwarz et al., 2014) in the studied 8 transects in the Bulgarian section of the Danube, the largest amounts of coarse-grained fractions (D50 and D84) are at Novo Selo, Batin, Silistra, as well as the research point above the Bulgarian sector of the river. The first two transects are within the range of potential spawning habitats identified by us based on catches of YoY specimens for the species *H. huso, A. ruthenus* and *A. stellatus*.

Based on sonar surveys, Schooley & Neely (2017) determined that substrates composed of gravel, stones and rocks (substrate hardness H-values ≥ 0.386) were most suitable for sturgeon spawning. Sonar-generated bottom hardness values for the potential habitats we investigated indicate that sites with suitable substrate (H ≥ 0.386) for spawning are available in the Baikal (643 – 640 rkm), Lom (746 – 745 rkm), Florentin (829 – 827 rkm), and Novo selo (839 – 835 rkm) areas. However, bottom samples collected by us to identify and validate the sonar-derived information on bottom hardness do not confirm the results obtained by Schooley & Neely (2017), as even at the sites with values ≥ 0.386 no gravel and stone substrate was detected. When comparing the bottom sample data and substrate information with the stated hardness values, the boulder, rock, and gravel sites we identified showed hardness data that corresponded to moderately hard or unsuitable substrates. This is most likely due to the specifics of the two studied rivers – in the Lower Danube there are more sections with sand, while in the river studied by Schooley & Neely (2017) the presence of sand is minimal, which may lead to differences in the reported sonar signal at various bottom sediments. Based on the obtained results, we can conclude that the bottom hardness data obtained from the sonar is not a good starting point for determining the substrate in the studied areas.

Sturgeon spawning grounds are often located in the main river bed, with a depth between 4 and 15 m considered optimal, and up to 20 m for *A. gueldenstaedtii*, and up to 40 m for *H. huso* (Holčik 1989). For some species such as *A. stellatus* and *A. ruthenus*, temporary floodplains of about 2 m depth can also be used for spawning (Billard & Lecointre 2001). All places surveyed by us using sonar in the Bulgarian part of the Danube have a depth of more than 6 – 13.1 m, and in the period September – October, when the hydrographic survey was carried out, the level of the Danube is low - between 144 and 117 cm. In the area of Vrav – Gomotartsi (839 – 815 rkm) from the Bulgarian coast, a section with a depth of between 8 and 12 meters and a substrate of stones and rocks has been delineated, and the registered section is not only suitable for the spawning of sturgeons, but is also a potential wintering habitat. Another such area is Cherkovitsa – Belene – Svishtov, with narrow sections from the shores or islands, with depths of 10 – 13.1 m and a substrate of stones and gravel.

The studied section in the Lom region (746 – 745 rkm), identified as a potential spawning ground based on catches of YoY sturgeons, is suitable in terms of the depth parameter (up to 7.7 m) as well as in terms of the nature of the bottom substrate dominated by gravel.

Three of the sites, at 651 – 647 rkm (Vadin), 515 – 512 rkm (Mechka – Pirgovo), 420 – 415 rkm (Pozharevo), during the sonar survey were identified as potential wintering habitats due to the suitable depth and conditions in these places. Such sites are usually used for wintering, with sturgeons using them to wait in close proximity to spawning habitats and to conserve energy during the winter (Friedrich 2012). These sites fall directly below the potential spawning sites we identified, further confirming that these sites may be used by sturgeons.

The other main factor for sturgeon reproduction is the appropriate current velocity (optimally > 1.0 – 2 $m.s^{-1}$) (Friedrich 2012, Staas 2017). When a predictive model was made for the section of the Danube in the Nikopol region (595 – 594 rkm), based on a complex analysis of depth, water levels, water outflow and current speed in the section for the period March – May (Dr. F. Penchev, personal communication) it was found that the appropriate current velocity was observed in a limited section from 800 m by 300 m to 1200 m by 400 m and within ten days, after which the current velocity was lower or the appropriate velocity is available in a very small area. Although Jakob (1996) determined that an adult female sturgeon needs an area of 300 m^2 to deposit her eggs, there is no valid information on the minimum habitat size at which reproduction occurs.

Despite numerous projects and studies of the Danube River (ICPDR 2007, 2019, Schwarz et al. 2014) a comprehensive picture in terms of bathymetry, substrate, current velocity, etc. parameters, is missing due to data fragmentation or unavailability resulting from its ‘classified information' status. This deprives us of the opportunity to build a complete map of suitable sturgeon spawning habitats in the Bulgarian section of the Danube.

The calculated spawning grounds of *H. huso*, *A. ruthenus* and *A. stellatus* are located in the river section from just below the Iron Gates II Dam to 150 – 170 km downstream. A second river section has been estimated as potential spawning ground for at least *A. stellatus* at rkm 535 – 600 (Vardim – Belene – Nikopol). Natural reproduction of four sturgeon species (*A. ruthenus, A. stellatus, A. gueldenstaedtii* and *H. huso*) still occurs in the Lower Danube but does not happen every year, and the number of registered young-of-the-year is very low, often single individuals. This very low level of natural recruitment cannot support the long-term survival of Danube sturgeon populations.

5.3 *Sturgeon diet and feeding habitats*

We established a significant dominance of the amphipod *O. obesus* and the mussel *C. fluminea* in the diet of the young sturgeons inhabiting the Danube. Two other major items are *Chelicorophium* spp. and chironomids. In the middle of the last century, amphipods were an insignificant part of the diet of sturgeons, while the most important food items were insects from the Trichoptera and Ephemeroptera (Rusev 1963). Representatives of Trichoptera were found in YoY *A. ruthenus* only in 2021 and in insignificant abundance, while representatives of Ephemeroptera were not found in the stomach contents of the studied sturgeons.

Species such as *Palingenia longicauda* and *Ephoron virgo*, which were an important part of the total productivity of the Danube River ecosystem in the 1970s, now belong to the most critically endangered species of Ephemeroptera in Europe (Bálint et al. 2012). This overall change in the invertebrate composition of the environment is the likely cause of the change in the diet of sturgeons. Rusev (1963) found that the species *P. longicauda* and *E. virgo* represented 39.86% of the food mass for *A. ruthenus*. Nowadays, *P. longicauda* covers only 2% of its former range (Bálint et al. 2012). After the 1970s, this species completely disappeared from the Bulgarian sector of the Danube (Russev 1987) due to changes in abiotic factors, regulated banks and organic pollution (Evtimova et al. 2019). Species of the *Hydropsyche* have been recorded in the environment (present study, Graf et al. 2015), but species of the genus were found in sturgeon stomach contents only in 2021. Although in the past *Hydropsyche* were of great importance for feeding *A. ruthenus* and the hybrids *A. ruthenus* × *A. stellatus* (Rusev 1963), today the relative importance of the genus is only 3.29% and 1.34% for the respective sturgeon species. Larvae of the aquatic insects Chironomidae occur in high frequency throughout the Danube River reach (present study, Graf et al. 2014), which is most likely the reason that their role has not changed as a common item of the sturgeon diet.

On the other hand, Ponto-Caspian crustaceans (e.g., *Chelicorophium* sp., *D. villosus* and *O. obesus*), which are native to the Lower Danube, show an increase in their numbers. In recent years, these species have successfully expanded their range to the upper reaches of the Danube (Borza et al. 2018, 2021). Although *O. obesus* is the most important food item for two of the studied sturgeon species (*A. stellatus*, *A. gueldenstaedtii*) and for the hybrids, in the samples studied by us it has a density of only 14 ind.m^{-2} for the entire Bulgarian section. Despite our results, a number of studies have confirmed the high abundance of *O. obesus* in the Danube (Borza et al. 2017, 2018), making it an easily accessible prey item for young sturgeons.

As for *Chelicorophium* species, which are the main items of the diet of *A. ruthenus* below 35 cm, an increase in density was also observed for the Lower Danube (Graf et al. 2014). According to Borza et al. (2018) the three species *C. sowinskyi*, *C. robustum* and *C. curvispinum* are found in different densities throughout the Danube River, with *C. curvispinum* being the most numerous (5,280 ind.m^{-2}), while *C. sowinskyi* was recorded with the highest abundance at 532 rkm. The specimens of *Chelicorophium* were found by us in higher numbers in several locations. Although the species *C. robustum* was more abundant in the bottom samples, it was not detected in the stomach contents of the sturgeons. This is most likely due to the smaller body sizes of *C. curvispinum* and *C. sowinskyi* species (Borza et al. 2018 b), which contributes to their easier ingestion by age 0+ sturgeons.

Recent studies have shown the strong decline of typical Danube mollusk species such as *Unio crassus* and *Theodoxus transversalis* and the invasion of alien species such as *C. fluminea* (Hubenov et al. 2013). In the studied sturgeons, the species *C. fluminea* was recorded as the main food item only in the adults' *A. ruthenus*. The consumption of specific species that occur in high abundance was also observed in a study of *A. ruthenus* feeding in a section between the two dams in the gorge of Iron Gate (Janković et al. 1994). Janković et al. (1994) attributed this to a shift in benthic faunal composition caused by dam dewatering. The mass distribution of *C. fluminea* in the Bulgarian site, with an abundance of up to 16,560 ind.m^{-2} in some localities (Hubenov et al. 2013), makes it an easily accessible food resource for adult *A. ruthenus*.

Based on the results obtained from the study of the macrozoobenthos in the Danube River, the most abundant food base for sturgeons was found in the regions of Vetren (396 rkm), Belene (576 – 578 rkm), Baikal (642 rkm), Tsibar (714 – 717 rkm) and Lom (746 rkm).

The diet for *A. stellatus* established by Rusev (1963) is significantly more diverse than the species recorded in the present study. Main prey *O. obesus* and chironomids dominated with 99.85% relative importance, while species of Ephemeroptera, Trichoptera were not found and mysids and *Chelicorophium* sp. are a very small part of the diet of *A. stellatus*. Data on the main prey, larvae of gammarids and chironomids, of young *A. stellatus* are available for the Volga River and the Kura River, but Oligochaeta, which are indicated for the Danube River by Reinartz (2002), were not found in the studied *A. stellatus*.

All collected *A. gueldenstaedtii* in 2019 originated from aquaculture and were restocked earlier that year, while the individual studied in 2021 was a naturally hatched specimen in the Danube, and no difference was observed in the organisms included in their diet. Zolotarev et al. (1996) indicated that juvenile *A. gueldenstaedtii* feed mainly on molluscs, which we did not detect in the stomach contents of the studied specimens. On the other hand, Rusev (1963) notes that *A. gueldenstaedtii* use for food species of Gammaridae, which includes the *O. obesus* that we found in the stomach contents of *A. gueldenstaedtii*. The high percentage of relative volume of the prey found in the stomach contents of the stocked *A. gueldenstaedtii* specimens indicates their successful adaptation to feeding in natural conditions. The young *A. gueldenstaedtii* successfully adapted to feeding in natural conditions and searched for food sources in the same places as the wild sturgeons. Our observations were similar to the made in the Danube Delta where restocked specimens were found to consume mainly Tubifex and gammarids (Paraschiv 2011, Cristea et al. 2016).

Despite the dominance of *C. fluminea* in adults' *A. ruthenus* and *Chelicorophium* spp. in the YoY specimens, in this species we observe the greatest diversity in the diet. Only the food contents of *A. ruthenus* displayed representatives of fish (*N. melanostomus*) and Simuliidae, which Reinartz (2002) indicated as part of the diet of the species.

Comparison of the seasonal aspects of feeding in *A. ruthenus* in the Danube River showed that the same groups of benthic fauna were part of the diet of the species throughout the year with some differences in prey proportions according to the season (Janković et al. 1994). Changes in the species composition of sturgeon prey over time observed in 2019 are most likely related to a change in the nutritional needs of the growing fish. Specimens of size class L1 (TL < 35cm) mainly consume *O. obesus*, *Chelicorophium* spp. and chironomids, while specimens of total body length over 35 cm (L2) consumed mostly *C. fluminea*. Rusev (1963) found the same relationship between body size of *A. ruthenus* and the size and diversity of food items. The importance of *O. obesus* increased in August, meanwhile the species was not found in stomach contents in June. A similar trend was observed in the abundance of chironomids. The species *C. fluminea* has a large share in the food of *A. ruthenus* adults in June, but shows a decreasing share in July. Due to the short period of the study (June – August), it cannot be said that the observed change in the food items during the months is a consequence of a seasonal change in the abundance of invertebrates, but rather it is a result of the studied sturgeon size groups. On the other hand, it is interesting to note that the abundance of *Chelicorophium* spp. was increased in the stomach contents of the sturgeons studied in 2021. For example, for YoY hybrids studied in August 2019, the only prey was *O. obesus*, while for specimens of the same size class studied in August 2021, the main prey was *Chelicorophium*. Such a trend was also observed in chironomids with a significantly increased percentage of their abundance in the stomach contents of the sturgeons studied in 2021. We assume that this change may be due to dredging activities carried out in the area of the village of Popina (about 405 rkm) in 2020, which led to the accumulation of sediments and a change of the substrate in the section in the Vetren area, and thus probably a change of benthic communities. This assumption is also supported by the data obtained by Moog et al. (2015), who found that sediment dredging in the Danube River strongly affected benthic invertebrates, with macroinvertebrate fauna in the dredged section recovering after about 235 days.

The overlap in the diet is significant for the species *A. gueldenstaedtii*, *A. stellatus* and the hybrids. Similar observations were made for *A. stellatus* and *A. gueldenstaedtii* in the Caspian Sea (Reinartz 2002). According to Rusev (1963), the greatest overlap is in the diet of *A. ruthenus* and the hybrid *A. ruthenus* × *A. stellatus*, which we did not observe, despite the increased amount of *Chelicorophium* spp. consumed in the studied YoY *A. ruthenus* and hybrids in 2021. The species *A. ruthenus* differs from other sturgeon species. The observed differences are most likely due to the body size and age of the studied fish. This is also confirmed by Rusev (1963), who points out that, although the *A. gueldenstaedtii* and *A. stellatus* use the same food items as the *A.*

ruthenus, an increased abundance of gammarids is observed in their diet. According to the same author, the species *P. abbreviatus* found in *A. gueldenstaedtii* and *H. hus*o has large body size, which is why it can only be consumed by adult *A. ruthenus* (Rusev 1963). This is also the reason for the observed difference in the diet of the YoY sturgeon we studied. The smaller sizes of *Chelicorophium* make them a more easily digestible resource for YoY *A. ruthenus* than *O. obesus* consumed by *A. stellatus* and *A. gueldenstaedtii*.

Both parasites found in the stomach contents of sturgeons *H. bidentatum* and *Pomphorhynchus* sp. have been recorded in the family Acipenseridae, which are the final hosts of these parasites (Emde et al. 2012, Moravec 2013). Third- and fourth-stage larvae and adults of *H. bidentatum* were found in all studied sturgeon species. According to Djikanovic et al. (2014), the diet of *A. ruthenus* includes members of the Nematoda, which, we presume, refers again to parasitic species.

The availability of food is an important parameter determining the suitability of habitats for sturgeons and other migratory fish species. According to Rochard et al. (2001), differences in the frequency of occurrence of prey items in the stomach of sturgeons reflect their distribution and abundance in the study region. The change in the species composition and the quantity of the macroinvertebrate fauna also indicate that changes have occurred in the bottom habitats. All changes affecting the riverbed influence to some extent the species structure and the amount of benthic invertebrates. Accordingly, this can lead to changes in both the composition of the diet and the locations of food for benthic fish. Access to the recently ingested food by sturgeons allows the identification of the feeding sites, which is useful for habitat research. Brosse et al. (2002) found that the gastric lavage method could recover organisms that had been ingested in approximately the last 2 hours. On the other hand, the ecological preferences of the organisms included in the sturgeon diet can help to identify parameters in feeding habitats.

According to the analyzed data on the autecological features of the macrozoobenthos organisms found in part of the sturgeon diet, it can be assumed how a potential feeding habitat would look hypothetically: the large number of macroinvertebrate species we found prefer the main part of the middle and lower river courses (hydromorphological preferences – river bed; river zoning: epi- and metapotamal and rillarial). The Danube in our entire section meets these conditions, with the potential habitats being limited to the river itself, without tributaries, spillways, canals, old riverbeds. The species found in the stomach contents are typical river inhabitants (preference for water course – rheophiles or rheo-limnobionts). In the Bulgarian section of the Danube, habitats with a faster current speed are very common, and this parameter coincides with the requirement that such a theoretical habitat should be located in the main part of the river. The preferred microhabitats of the macrozoobenthos, which has become a food source for sturgeons, are bottom substrates of lithal (gravel over 2 cm), partly phytal and sand. This result suggests that sturgeons feed preferentially on such a substrate, and its presence is determined by a faster current speed (delayed sedimentation processes), that is, the parameter corresponds to the above-mentioned hydromorphological features. The macrozoobenthos organisms, which are included in the sturgeon diet, are sessile or hemi-sessile (type of locomotion – without floating and swimming, the most slowly mobile, followed by crawling, walking, burrowing). This type of movement is directly related to the above-listed characteristics of the theoretical feeding habitat – in the river section with a greater water speed and on a hard and coarse substrate, mostly slow-moving and fixed benthos is expected. In summary, probably the most suitable feeding habitat for sturgeons, according to the autecological features of the macrozoobenthos that became food source for the fish, corresponds to the following description: it is a main river section, characterized by a moderate current speed, not allowing sedimentation processes, but not requiring a physical effort for the fish to overcome. It cannot be a channel, shoal, where there are silt deposits and a slow current. Such potential habitats exist in the Bulgarian sector of the Danube in the regions of Novo selo (839 – 835 rkm), Lom (746 – 745 rkm), Tsibar (717 – 714 rkm), Belene (576 – 578 rkm), Vetren (399 – 393 rkm), etc.

It can be concluded that the sturgeon feeding habitats are still mainly represented by gravel-stony bottoms, but the importance of the sandy substrate is also increasing. In view of the river channel and littoral zone preference of most of the organisms included in the sturgeons diet, we can assume that there is a slight shift in feeding habitat from the littoral areas to deeper parts of the river. The observed feeding behavior is an adaptation to the diversity of food resources in the river, while at the same time no specialization to a particular prey was observed in the sturgeon.

For now, despite great research efforts, the only habitat for growth and aggregation of sturgeon fish along the Bulgarian section of the Danube is located at 396 rkm near the village of Vetren, which is also a very important habitat for many other endangered Danube fish species.

6 Conclusions

1. The natural restoration and replenishment of sturgeon populations in the Lower Danube demonstrates a steady downward trend.
2. The four sturgeon species *A. ruthenus, A. stellatus, A. gueldenstaedtii* and *H. huso* still breed naturally in the Bulgarian section of the Danube. The potential breeding habitats of the sturgeon fish in the Bulgarian section of the Danube River have different locations for the individual species:
   - For the species *H. huso*: 750 – 800 rkm (Lom – Vidin);
   - For the species *A. ruthenus*: 700 – 763 rkm (Kozloduy – Archar) and a second at section 763 – 863 rkm (Archar – Iron Gate II dam);
   - For *A. stellatus*: 700 – 763 rkm (Kozloduy – Archar) and a second at section 535 – 600 rkm (Vardim – Belene – Nikopol);
   - For the species *A. gueldenstaedtii*: 680 – 700 rkm (Oryahovo – Kozloduy).
3. The species *A. ruthenus* and *A. stellatus* share the same spawning grounds and have similar breeding behaviour as evidenced by the establishment of hybrids between them.
4. The change in the diet of young sturgeons in the Lower Danube is the result of a decrease in the diversity of species that make up the food spectrum of sturgeons, compared to data from 60 years ago, as well as a change in the main food components with a shift from insects to gammarids. These changes are initiated by the introduction of alien species and the extinction and decline of native invertebrates as a result of anthropogenic impacts.
5. A preferred type of substrate in sturgeon feeding habitats is gravel-rock. At the same time, however, the importance of the sandy substrate is increasing, due to a change in the predominant groups of macrozoobenthic organisms, as well as in the availability of food sources.

6. The composition of the macrozoobenthic communities in the Bulgarian section of the river, although changed over the years, represents a suitable food base for sturgeon fish.
7. Stocked sturgeons of the species *H. huso*, *A. ruthenus*, *A. gueldenstaedtii* adapt to the conditions in the river and the Black Sea, and have a good growth rate. Restoked fish feed in the same places as wild sturgeon.

7 Contributions – scientific and applied

1. Up-to-date information on the status of sturgeon species and their habitats in the Bulgarian section of the Danube River has been collected. Based on the established continuing trend of reduction of sturgeon populations, a recommendation was made through the relevant institutions (MoEW, MAFF) to extend the ban, as 5-year ban periods are too short for the recovery of sturgeon populations, given their long life-cycle, late sexual maturity and specific reproductive requirements.
2. Sturgeon bycatch in the Danube River and the Black Sea was studied for the first time after the introduction of a sturgeon fishing ban in 2011; the importance of working with local fishing communities was also assessed. Recommendations have been made to the relevant institutions (Executive Agency for Fisheries and Aquaculture) regarding information campaigns on the status of sturgeons and joint work with fishing communities in the Danube and Black Sea regions in order to report by-catch and offer complete information on the status of populations. In addition, a recommendation was made to increase the control over sturgeon poaching, which still occurs in the Lower Danube.
3. Information was collected on the behaviour of stocked cultured sturgeons, and their growth rate after release into the river. The information is valuable for monitoring the sturgeon's ability to continue their life cycle in the natural water body and confirms the effectiveness of activities supporting the maintenance and recovery of sturgeon populations in the Danube. Based on the established tendency of decrease in the populations and the information on the behaviour of stocked cultured sturgeon fish, a recommendation was made through the relevant institutions (Ministry of the Environment and Waters) to support the recovery of populations through stocking.

4. For the first time, data are presented regarding length-weight relationships and relative condition factor for young-of-the-year and juvenile sturgeon specimens of the species *A. ruthenus*, *A. stellatus* and *A. gueldenstaedtii* from the Bulgarian section of the Danube.
5. The speed of movement of young-of-the-year sturgeons of the species *H. huso*, *A. ruthenus*, and *A. gueldenstaedtii* during their migration to the Black Sea was determined. The impact of hydrological regime changes resulting from anthropogenic activities on the migratory behaviour of young sturgeons was determined, and a tenfold increase in the speed of movement of young sturgeons was reported.
6. The period during which sturgeons breed in the Bulgarian section of the Lower Danube was established, based on data on temperature and water level. Based on this model, recommendations have been prepared to the relevant institutions to change the research strategy and supplement the methodology for monitoring sturgeons in the Danube River and the methodology for assessing the status of fish species. A recommendation has also been made to unify the methodologies for monitoring and assessing the status of fish species in the countries of the Danube River region in order to provide comparability and completeness of data regarding the status of sturgeons from the Danube watershed.
7. The potential spawning habitats of the four sturgeon species *H. huso*, *A. ruthenus*, *A. stellatus* and *A. gueldenstaedtii* in the Bulgarian section of the Danube have been determined, on the basis of which specific spawning areas can be located. The main parameters (depth, substrate and food base) in potential sturgeon habitats have been studied, which indicate the presence of suitable places for sturgeon reproduction and feeding in the Bulgarian section of the Danube River. This information is important for taking environmental protection measures.
8. The current diet of three sturgeon species *A. ruthenus*, *A. stellatus* and *A. gueldenstaedtii*, as well as sturgeon hybrids, was studied, also a

change was established in the diet of sturgeons in the Bulgarian section of the Danube River.

9. For the first time, a complete ecological characterization was made of the macrozoobenthos as a food resource in the feeding habitats of the sturgeons in the Bulgarian section of the Danube River.
10. At rkm 396 of the Danube River (in the Vetren area) a habitat for growth and feeding was identified, which the small fish of the four sturgeon species *H. huso*, *A. ruthenus*, *A. stellatus* and *A. gueldenstaedtii* preferentially choose for searching food and shelter. A proposal was made to the Ministry of the Environment and Waters for a new protected area in the vicinity of the village of Vetren, Silistra municipality, which has an area of 2884 decares, with the aim of protecting the habitats of sturgeons. The proposed regimes according to Art. 34 of the Law on the Protected Territories include prohibitions on: extracting aggregates from the river bed of the Danube River; dredging the bottom, depositing dredged mass and disrupting the natural sediment transport; waste disposal; discharge of untreated wastewater; disruption of the natural hydro-morphological processes in the river, including the construction of hydrotechnical facilities and activities that would lead to the redirection and delay of water flows and the closing of the side river flow feeding the Srebarna reserve; construction of facilities leading to drainage or lowering of the water level; other activities that may lead to the destruction of species or disturbance of the natural state of the species communities. As a result, the Ministry of the Environment and Waters issued an order for the declaration of the "Esetrite - Vetren" protected area in this section of the Danube.

Publications related to the dissertation

- Mihov S., **Margaritova B**., Koev V. (2022) "Downstream migration of young-of-the-year sturgeons (Acipenseridae) in the Lower Danube River, Bulgaria", Biodiversity, https://doi.org/10.1080/14888386.2022.2099462;
- **Margaritova B.**, Kenderov L., Dashinov D., Uzunova E., Mihov S. (2021) Dietary composition of young sturgeons (Acipenseridae) from the Bulgarian section of the Danube River, *Journal of Natural History*, 55(35-36): 2279–2297 https://doi.org/10.1080/00222933.2021.2005838;
- **Margaritova B.,** Uzunova E. (2020) Length–Weight Relationships and Relative Condition Factors of Three Sturgeon Species (Acipenseridae) From the Danube River, *Ecologia Balkanica*, vol. 12, issue 2, pp. 197-201;
- **Margaritova, B.** (2021) "Transboundary measures for the conservation and restoration of sturgeon fish in the Bulgarian section of the Danube River." Interdisciplinary Readings, Volume 2: Waters and Biodiversity, University Edition "St. Clement of Ohrid", pp. 113-128.

Participation in international and national conferences:

- **Margaritova B**., 2020. “Restocking of sturgeon species (Pisces: Acipenseridae): an important tool in conservation management in the Lower Danube River”, International Scientific Conference on Restoration of Conservation-Reliant Species and Habitats, 6 November 2020, Sofia, Faculty of Biology;
- **Margaritova B**., 2019. “Key sturgeon habitats - research approaches and conservation measures”, International Capacity Building Conference for youth in Wildlife Research and Conservation, Tbilisi, Georgia, October 3-4, 2019;
- **Margaritova, B**. 2019. Conservation and recovery of sturgeon fish in the Bulgarian section of the Danube, doctoral seminar, May 17-19, 2019, Kyustendil, Bulgaria;
- **Margaritova B.**, Dashinov D., Kenderov L., Uzunova E. 2019. Application of a microinvasive method for the examination of the gastric content of sturgeon species (Pisces: Acipenseridae) from the Danube River“, Youth Scientific Conference "Kliment's Days" 8-9 November, Sofia, Bulgaria.

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Protocols number 13 / 01.03.2014 and 12 / 25.02.2019 for permits for the fish handling methods used, issued by the Executive Agency for Fisheries and Aquaculture, Bulgaria.