Life+ Alosa alosa Irstea report 3: Actions C1 - D7 July 2013-June 2014

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Table of contents

Abstract

This report presents works managed by Irstea, Aquarium La Rochelle and Borea Team concerning the set up of an ex situ rearing method for juveniles Allis shads *Alosa alosa*, and the monitoring of sexual maturation. Because of biomass increase a second biological filter has been installed in April 2014. Mortality was higher during this period because of bacterial and kidney diseases. Histological analysis of samples collected in June 2013 showed a normal gonadal development. A third set of samplings for the monitoring of sexual maturation was carried out in June 2014.

Résumé

Ce rapport présente les travaux conduits par Irstea, Aquarium La Rochelle et l'équipe Borea dans le cadre de la mise au point d'une méthode d'élevage ex situ de juvéniles de grande alose *Alosa alosa* et du suivi de la maturation sexuelle. L'augmentation de la biomasse a conduit à l'installation d'un second filtre biologique en avril 2014. La mortalité a été plus forte en 2013 – 2014 à cause de pathologies bactériennes et de nodules calcaires dans le rein. Les analyses histologiques des prélèvements effectués en juin 2013 ont montré un développement normal des gonades. Une troisième série de prélèvements a été réalisée en Juin 2014.

Monitoring of the 2011 batch

1.1 Origin of the batch

This batch arrived in Aquarium La Rochelle on 24 May and 25 October 2011. These 2 groups were put together in December 2011, with a total number of 602 juveniles. Salinity was increased from 10 to 30 ‰ during the course of December 2011 (a one week transfer).

1.2 Rearing conditions

Fish were in a 8 m^3 tank from September 2012 (Figure 1). The net put to prevent fish to jump outside was not totally effective. The system was modified in October 2013. General rearing conditions were not modified (Figure 2). Mean water temperature has been increased from 17.6 ± 0.3 °C during the previous period to 19.3 ± 0.4 °C in order to obtain a better growth. Mean salinity was 30 ± 0.9 ‰, ie two points less from the previous period. Photoperiod was constant at 12/12.

Figure 1: The 8 m^3 rearing tank with the new net to prevent escapements.

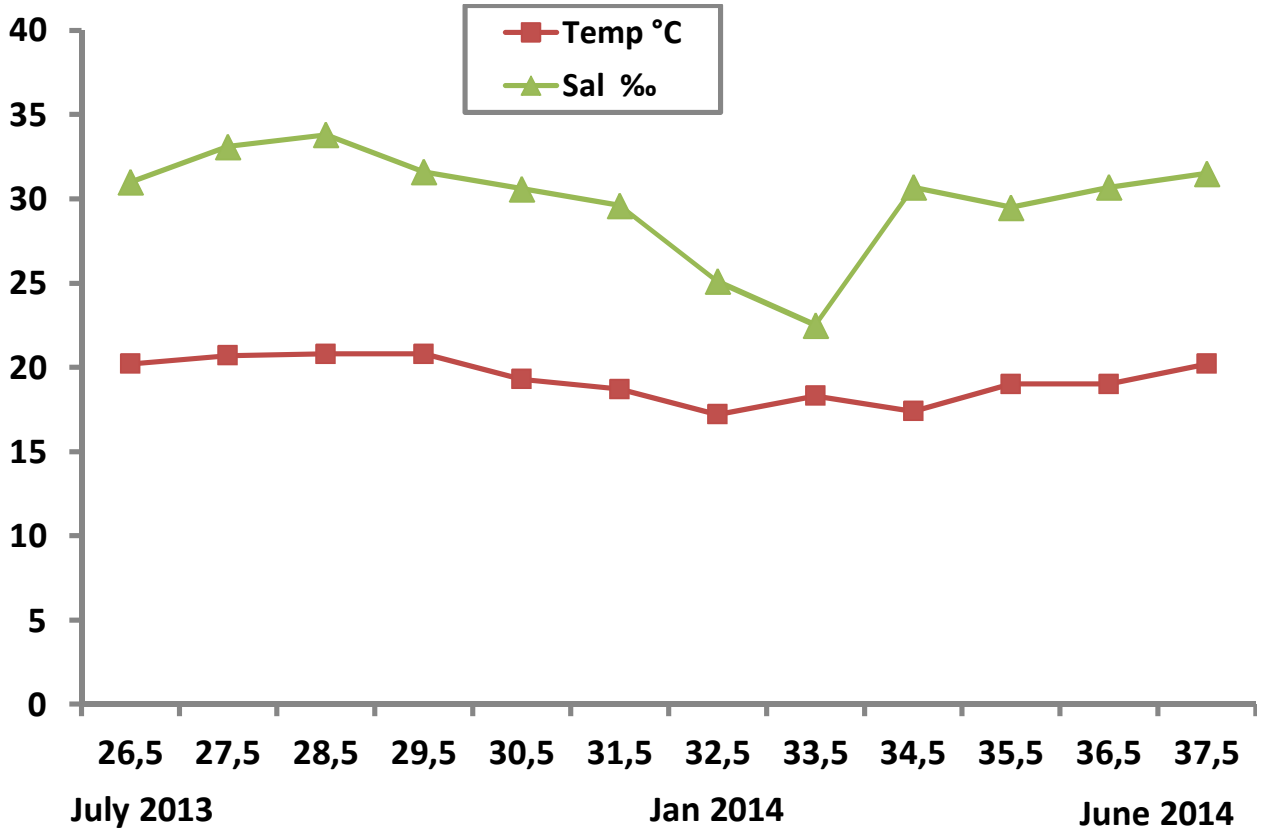


Figure 2: Monitoring of the temperature and the salinity from July 2013 to June 2014

Because of the biomass increase, the biological filtration capacity must be increased too. The nitrite concentrations recorded (0.3 $mg.l^{-1}$) proved that the efficiency limit of the biological filter has been reached. A second biological filter system has been put in at the end of April 2014 (Figure 3) with a degasing procedure at the end of June 2014.

Figure 3: The biological filtration system installed in April 2014.

Current speed was measured in March 2014. At 20 cm from the inner wall and at mid depth, the current was 13.4 ± 0.4 cm.s^{-1}.

1.3 Feeding

Fish were fed with artificial feed (3.5 mm floating pellets) and frozen zooplankton (krill), the same feeding regime as the previous period.

A total amount of 123.63 kg of pellets and 39.90 kg of krill have been distributed during this period. The mean feeding ratio was 1.05 and 0.34 for the artificial feed and zooplankton respectively. A theoretical feeding ratio has been calculated according assessment of intermediate weight gain during the period and food distributed daily (Figure 4). The feeding ratio decreased from December 2013 to prevent degradation of water quality because the biological filtration capacity limit was reached regardless to biomass. Once the additional system was installed, the feeding ratio has been increased.

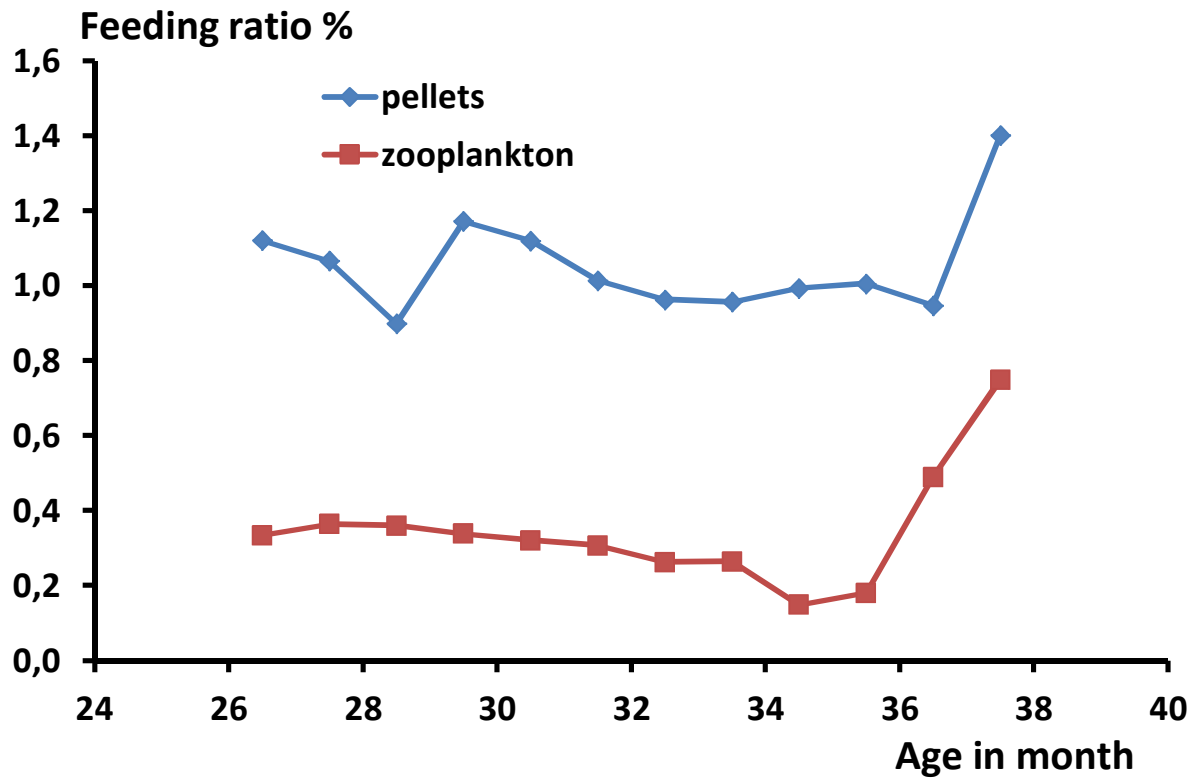


Figure 4: Theoretical feeding ratio during the period 2013-2014

1.4 Mortality

Mortality was higher during this third year of rearing (Figure 5). Monitoring of the survival is only based on natural mortality; fish collected for biological sampling or fish transferred to aquarium exhibition or in freshwater for test, were not counted. Natural mortality corresponded to 95 fish. Two main mortality phases were observed. The first during November – December 2013 correspond to a parasitic and bacterial pathology which was stopped following a 6 days treatment. The second was observed from April. Dead fish were examined and presented calcium nodules in the kidney ("nephrocalcinose") due to too low pH and hardwater (Figure 6). Mortality was stopped thanks to the addition of the new biological filtration and degasing system. At the end of June 2014, 292 fish remained in the tank.

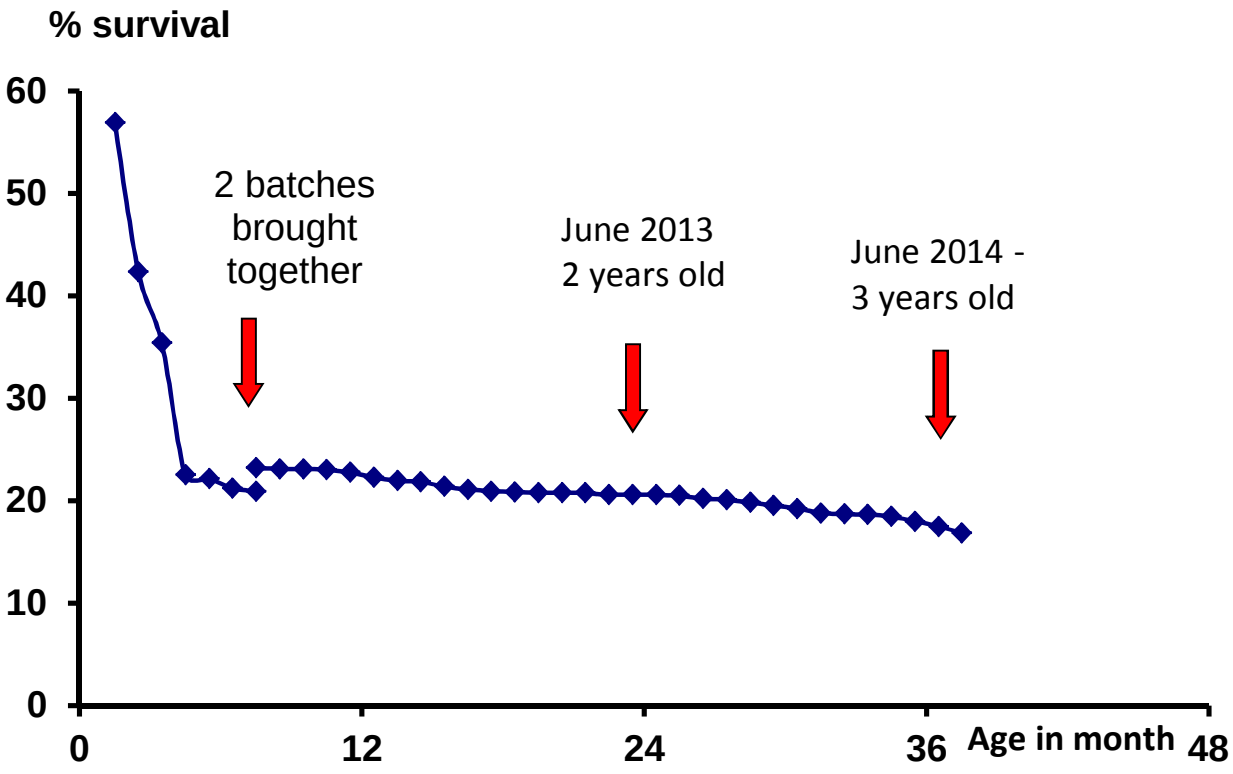


Figure 5: Survival after 3 years of rearing

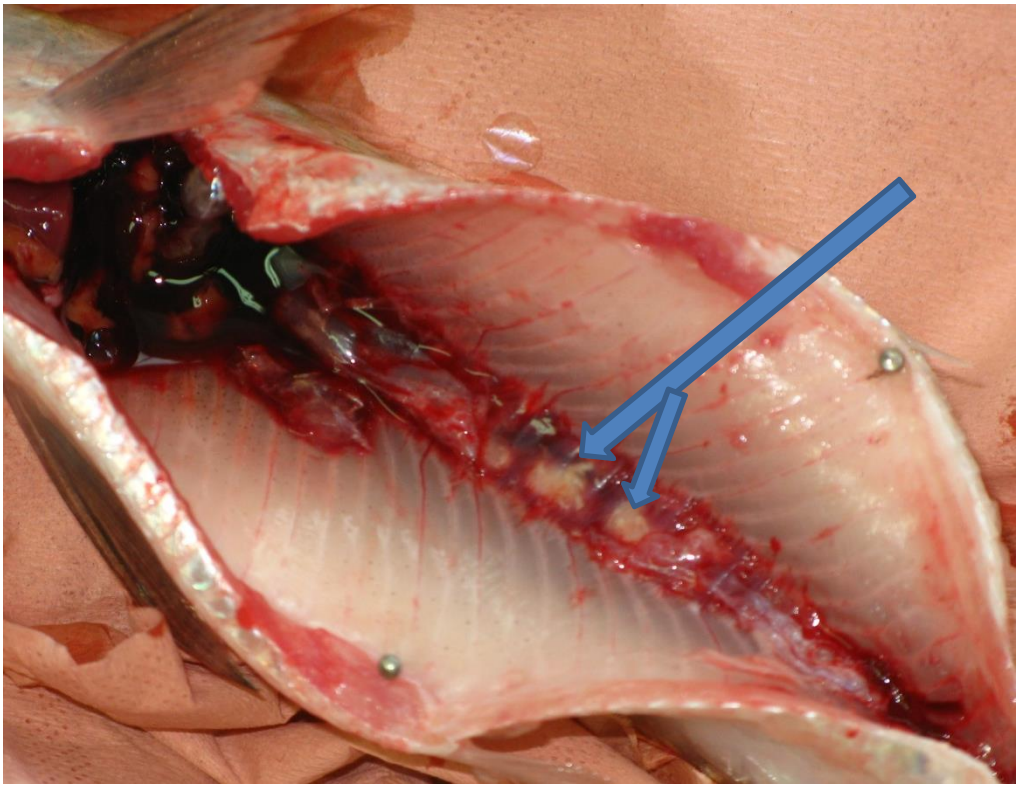


Figure 6: Presence of calcium nodules in the kidney (arrows)

1.5 Rearing density

Assessment of the rearing density from January 2012 is plotted in Figure 7. Rearing density reached 4.8 $kg.m^{-3}$ in March 2014, and was reduced to 4 $kg.m^{-3}$ at the end of June following mortality and samplings. Following veterinarians' opinions, rearing density in this kind of experimental rearing should not be higher than 2 $kg.m^{-3}$ to avoid pathologies and to obtain a correct growth. Following this advice, a second rearing tank will be installed in the end of 2014.

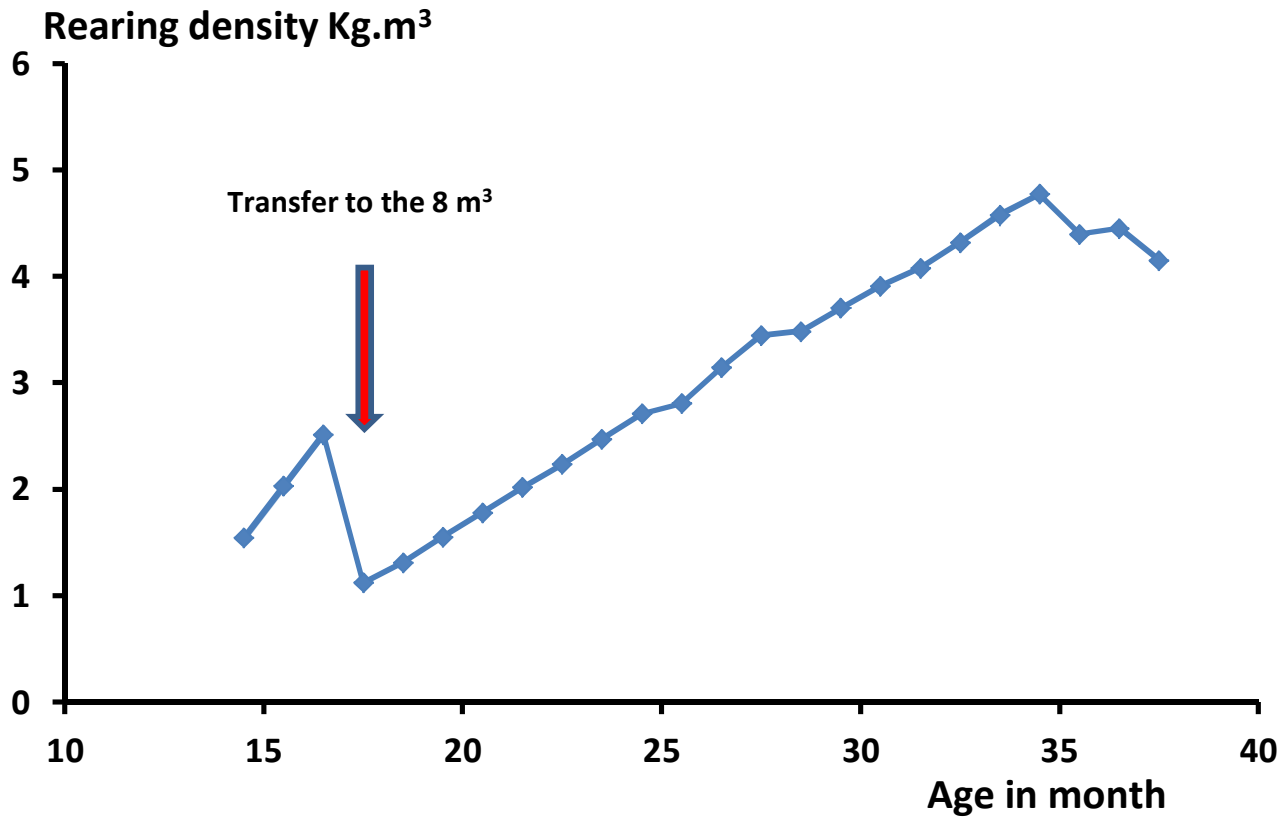


Figure 7: Rearing density from January 2012 to June 2014

1.6 Growth

The growth until June 2014 is represented in Figure 8. Specific growth rate (SGR, % body length day^{-1}) is 0.07 for length and 0.22 for weight between June 2013 and June 2014.

Food efficiency (or food conversion ratio) is basically assessed from biomass gain and dry food distributed

FCR = Food distributed / biomass gain

In our case, around 25% of the food distributed is frozen food, ie with a high level humidity, which doesn't allow comparison with aquaculture data. Moreover some fish (86) were removed from the rearing experimentation for samplings or aquarium exhibition which leads to a bias in the calculation. Considering these comments, food conversion ratio calculated during the third year of rearing is 16.3, so 16.3 kg of food (dry and moist food) is necessary to product one kg of fish.

Length – weight relationship was calculated from the beginning of the rearing. The relationship is plotted on Figure 9.

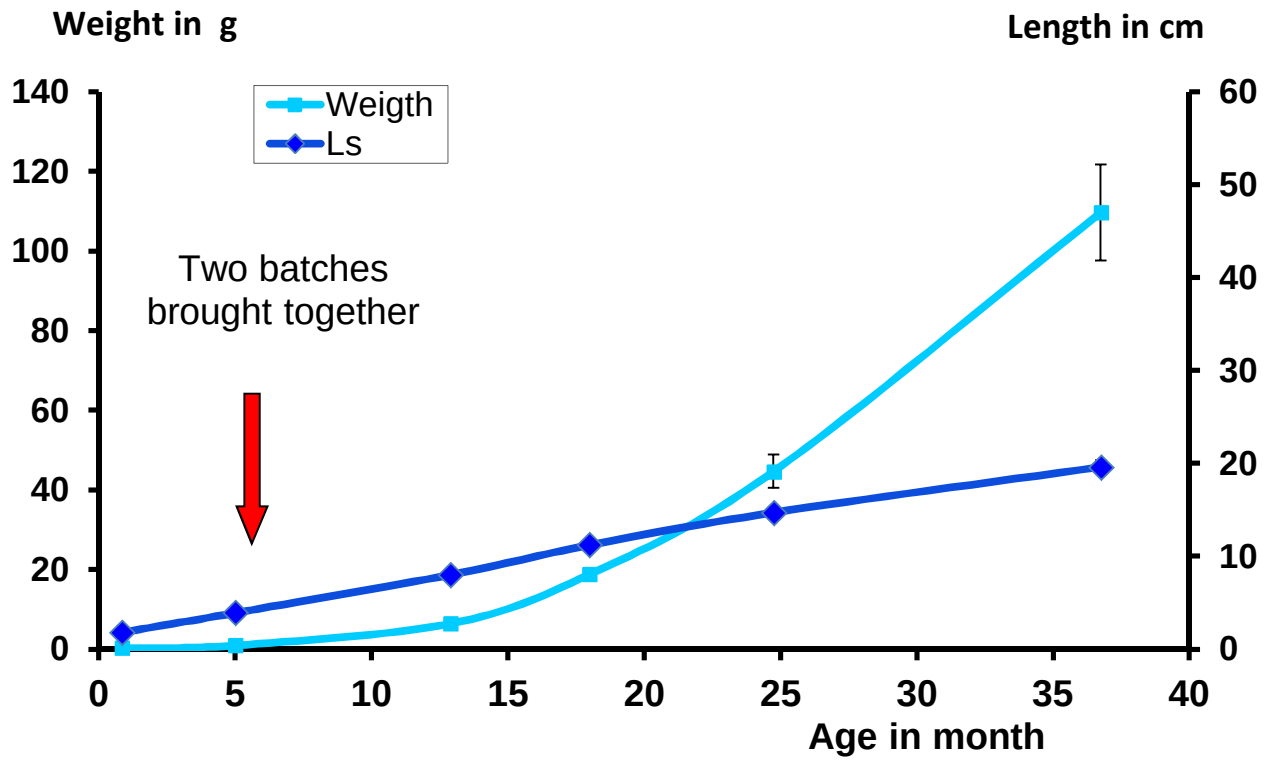


Figure 8: Linear and weight growth until June 2014

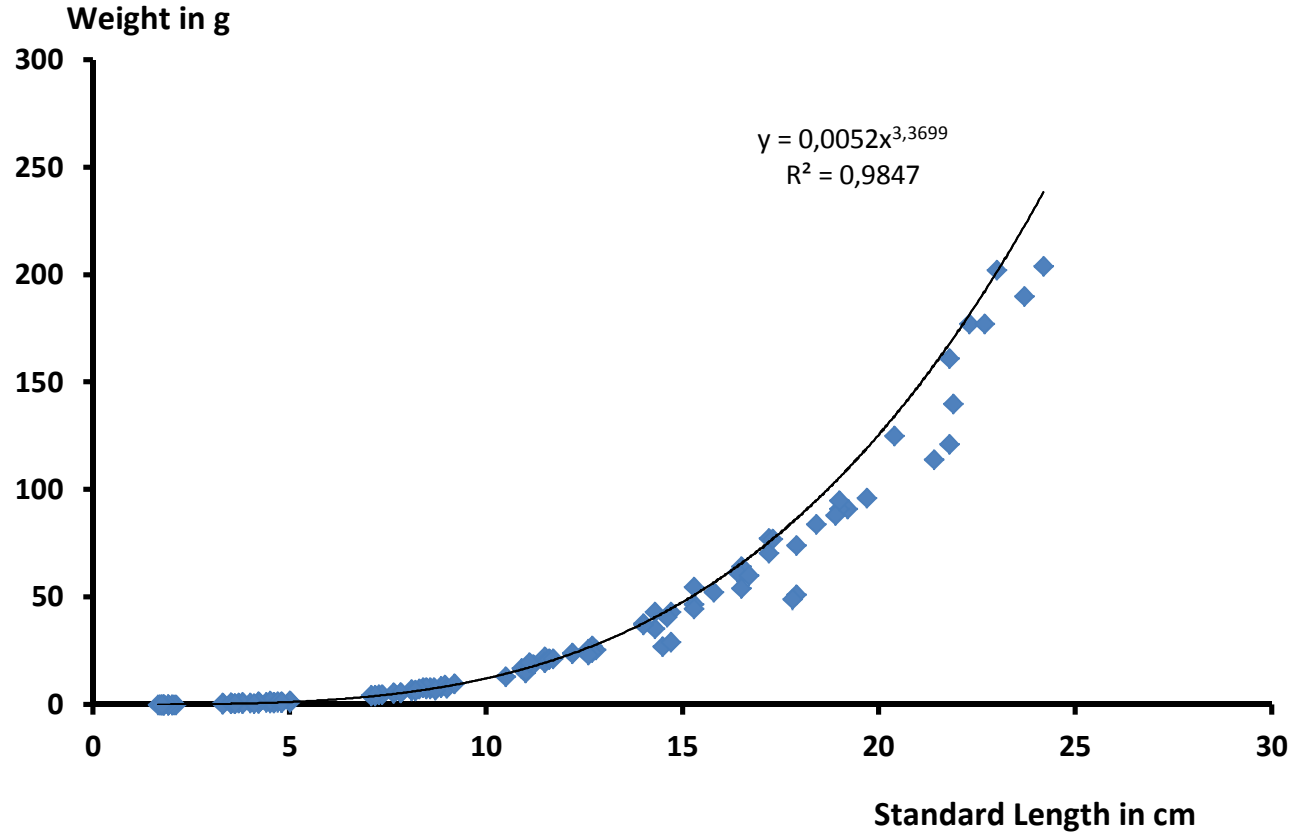


Figure 9: Length-weight relationship

Simulation of freshwater migration

2.1 Objective

Between 3 and 6 years, adult Shad leave the ocean to enter in the estuary and river to reach the spawning grounds and spawn. During this migration, Shad switch therefore from a salted to a freshwater environment.

In the C1 'ex situ stock' action framework, the aim is to bring to maturity a lot of Shad reared from the larval stage. In order to move closer the biological cycle of the species, it is therefore necessary to simulate the migration of adults to the spawning grounds by transferring the fish in fresh water.

2.2 Protocol

The transfer has involved 38 randomly caught Shad. In the case of mortality due to handling, there will be possibility to replace the dead during 7 days after the transfer.
Beyond 10 deaths during the first week, a rapid consultation of the three partners will decide on the continuation of the experimentation of transfer.
The transfer was managed in early April, with 3 year- old fish, with a gradual transfer on 2 weeks.
The objective was to maintain the fish for 2 months in fresh water.
The fish were transferred into a 3 m^3 tank (2 m diameter).

The decrease of salinity was gradual and continuous, by adding freshwater during the day, to obtain a decrease of approximately 1‰ per day. At the end of the first week of transfer, salinity was around 15‰. During the second week the freshwater input was increased to reach 0‰ at the end of the week.
During the freshwater phase, limited inputs of sea water were achieved (up to maximum 5‰) to prevent possible development of diseases (fungal or bacterial).

The temperature was planned to increase just before the sampling phase (which would correspond to the reproductive phase), with a passage from 20-21-22-23 ° C.

The current speed was maintained around 15 $cm.sec^{-1}$.
The duration of the light phase was also increased according to the following scheme:
In April: photoperiod 12N / 12L (from 7 h to 19 h)
In may: photoperiod 11N / 13L (from 7: 00 to 8: 00 pm)
In June: photoperiod 10N / 14L (from 07: 00 to 21: 00)

Feeding was maintained at least at the beginning of the transfer. The batch was observed to detect loss of appetite (fish do not eat during the migration phase). As food intake decreased, feeding was reduced, or even stopped.

2.3 Results

With only 4 dead, juvenile shads have well withstood transfer to freshwater (4 more deaths following injuries with an air diffuser).
Majority of shads seemed to rapidly stop feeding.
By the end of April, level of NO_2 increased because of the death of bacterial biomass in the biological filter (because of freshwater), which involved the progressive death of all fish.

2.4 Conclusion

Three year-old shads can tolerate transfer from seawater to freshwater within two weeks, without significant mortality.

Next year the test will be manage again, with a biological filtration system allowing to maintain good water quality in freshwater.

Monitoring of the sexual maturation (Borea Research Unit)

3.1 Histological analysis of the gonads of two year old shads.

Twenty shads had been sampled in June 2013.

3.1.1 Tissue sampling and histological procedures

Twenty two-year old shads had been sampled during the previous reporting period (June 2013, see 2nd Report). At two year of age, their body length ranged between 11.7 and 17.9 cm (SL) and their body weight between 21.2 and 77.3 g.

Gonads could be recognized by eye along the body walls, during the body dissection. Ovaries were larger than the testes and with a light orange colour, while testes had a white colour (see Photos, 2nd Report). Gonads were dissected out and quickly placed in freshly prepared Bouin's fixative solution, for histological analyses.

Histological procedure was performed as described in the 2nd Report.

3.1.2 Histological determination of gonad sex

Histology confirmed the visual sex determination, except for two fish (n° 38 and 42), for which the histological samples collected did not include the gonads. These two fish were removed from the analyses.

Altogether, histological analysis of the gonads could be successfully performed for 18 two-year old shads, and revealed that they corresponded to 9 females and 9 males.

3.2 Histological determination of gonadal stage

3.2.1 Females

Histology of the ovaries showed the presence of numerous primary oocytes, characterized by a dense ooplasma, and a large nucleus with many perinuclear nucleoli (for example, see Photos 10 and 11: female n° 40). This indicates that two year-old females are at the primary oocyte stage. As compared to one-year-old females (see 2nd Report), the oocytes of two-year old females are enlarged, but still at the primary oocyte stage.

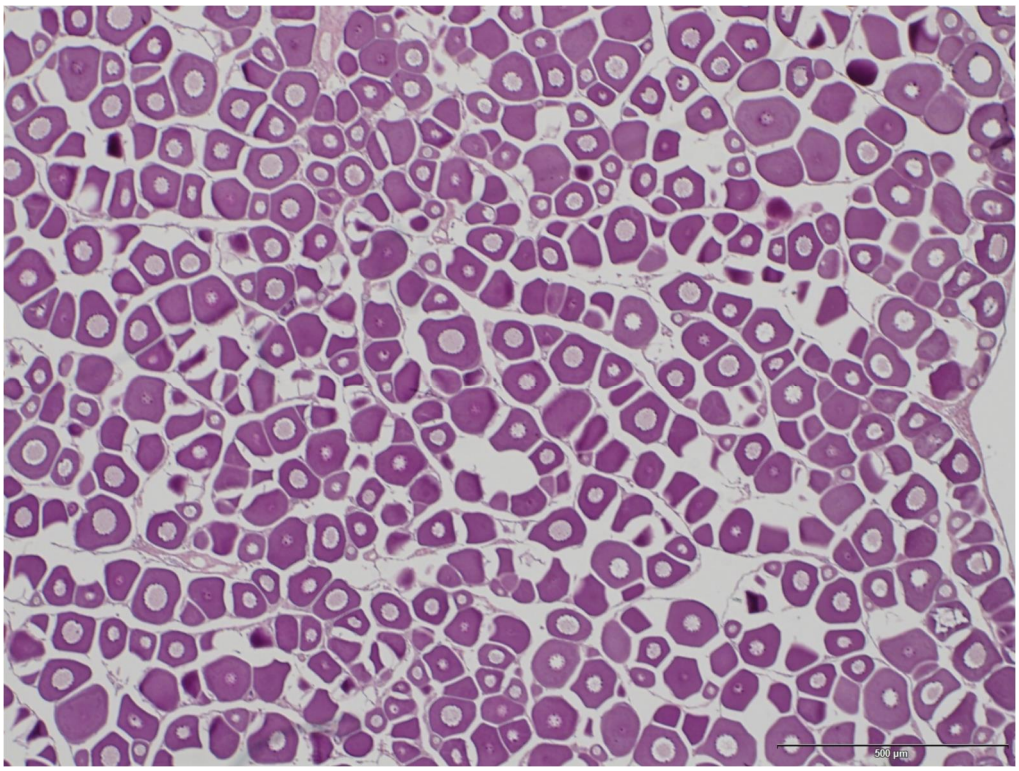


Figure 10: Histological section of ovary from two-year old female *Alosa alosa* (Female n°40; x 4) - Numerous primary oocytes are observed.

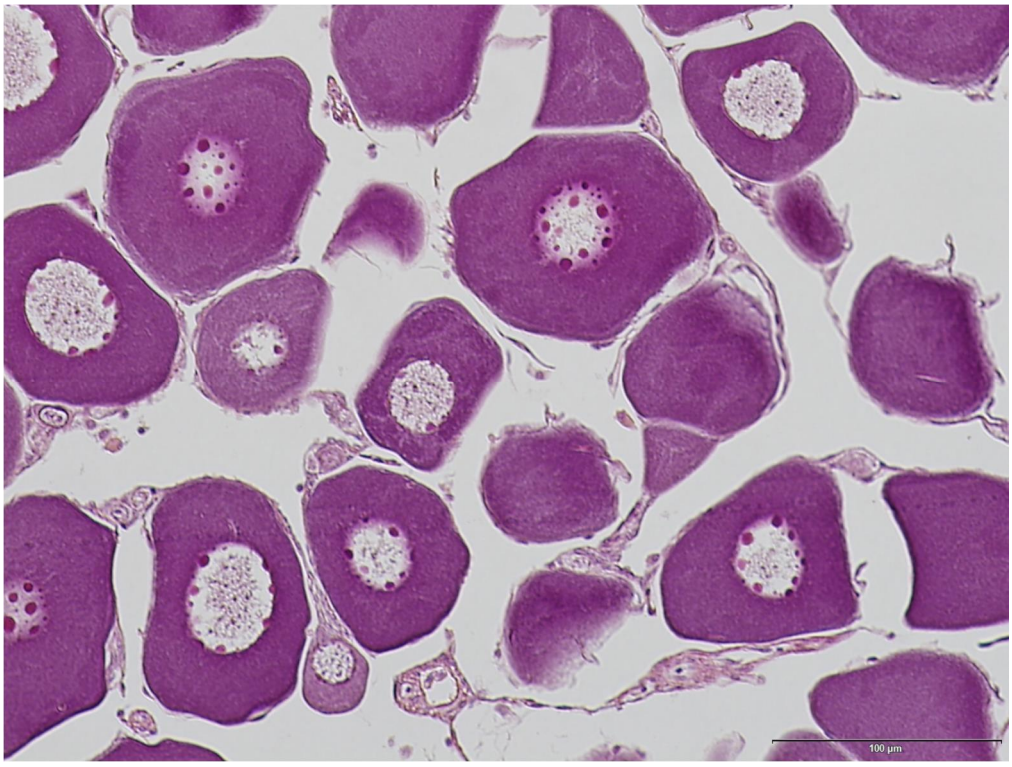


Figure 11: Histological section of ovary from two-year old female *Alosa alosa* (Female n°40; x 20) Primary oocytes with dense ooplasma. and perinuclear nucleoli are observed.

The presence in the ooplasma of a few lipid vesicles, indicating the initiation of the lipid vesicle stage, could be observed only in one fish (see Photo 12: female n°31).

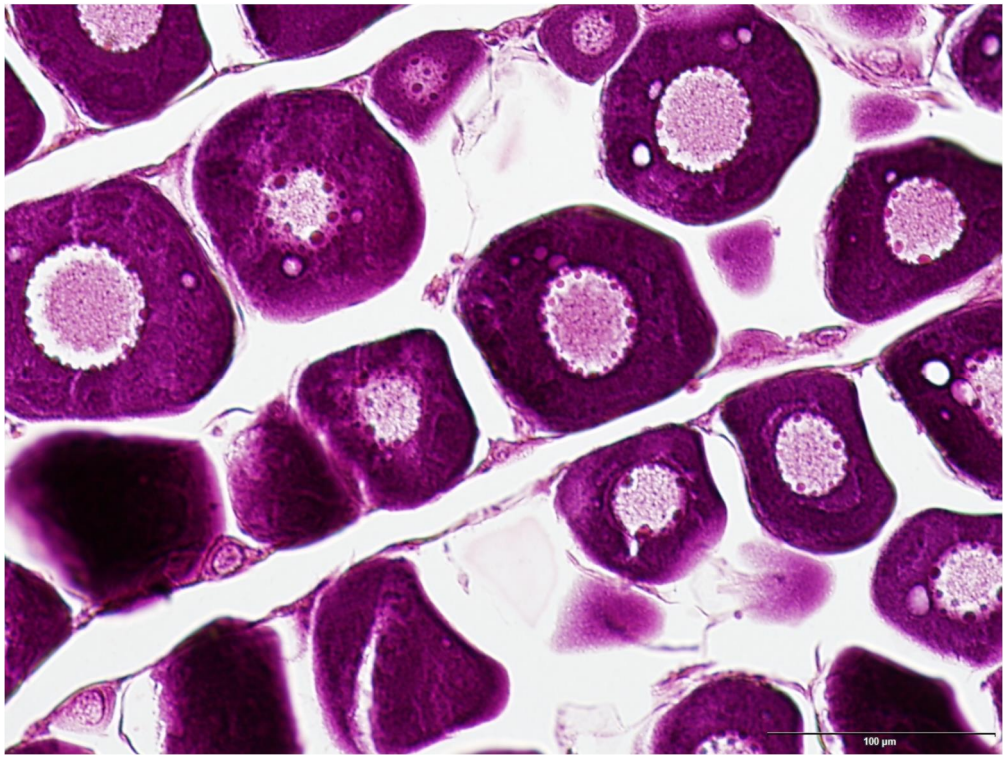


Figure 12: Histological section of ovary from two-year old female *Alosa alosa* (Female n°31; x 20) Primary oocytes with dense ooplasma and a few lipid vesicles, and perinuclear nucleoli are observed.

Measurement of oocyte diameters

The diameter of the 10 largest primary oocytes was measured for each female. The mean diameter ranged between 93 ± 3.4 (SEM) µm to 137.7 ± 4.9 µm according to fish (Table I). The mean diameter of the largest oocytes of two-year old shads was 109.8 ± 4.3 (SEM) µm.

As compared to one-year old fish (See 2nd Report: 36.7 ± 4.6 micrometers) these results indicate a 3 fold increase ($P< 0.001$) in the primary oocyte diameters in two-year old fish, which reflects the progress of primary oocyte growth (Figure 13).

Table I: Results of oocyte diameter measurements

	Mean diameter µm	Standard deviation (SD)	Standard error (SE)	Length (Ls) cm
N°26	108.3	15.3	4.8	16.5
N°29	111.0	13.9	4.4	14.3
N°31	113.0	10.9	3.5	15.3
N°32	96.6	9.0	2.8	14.7
N°34	105.9	11.7	3.7	11.7
N°35	96.6	9.0	2.8	17.9
N°37	116.6	10.6	3.3	15.8
N°39	93.4	10.8	3.4	14
N°40	105.4	9.9	3.1	14.3

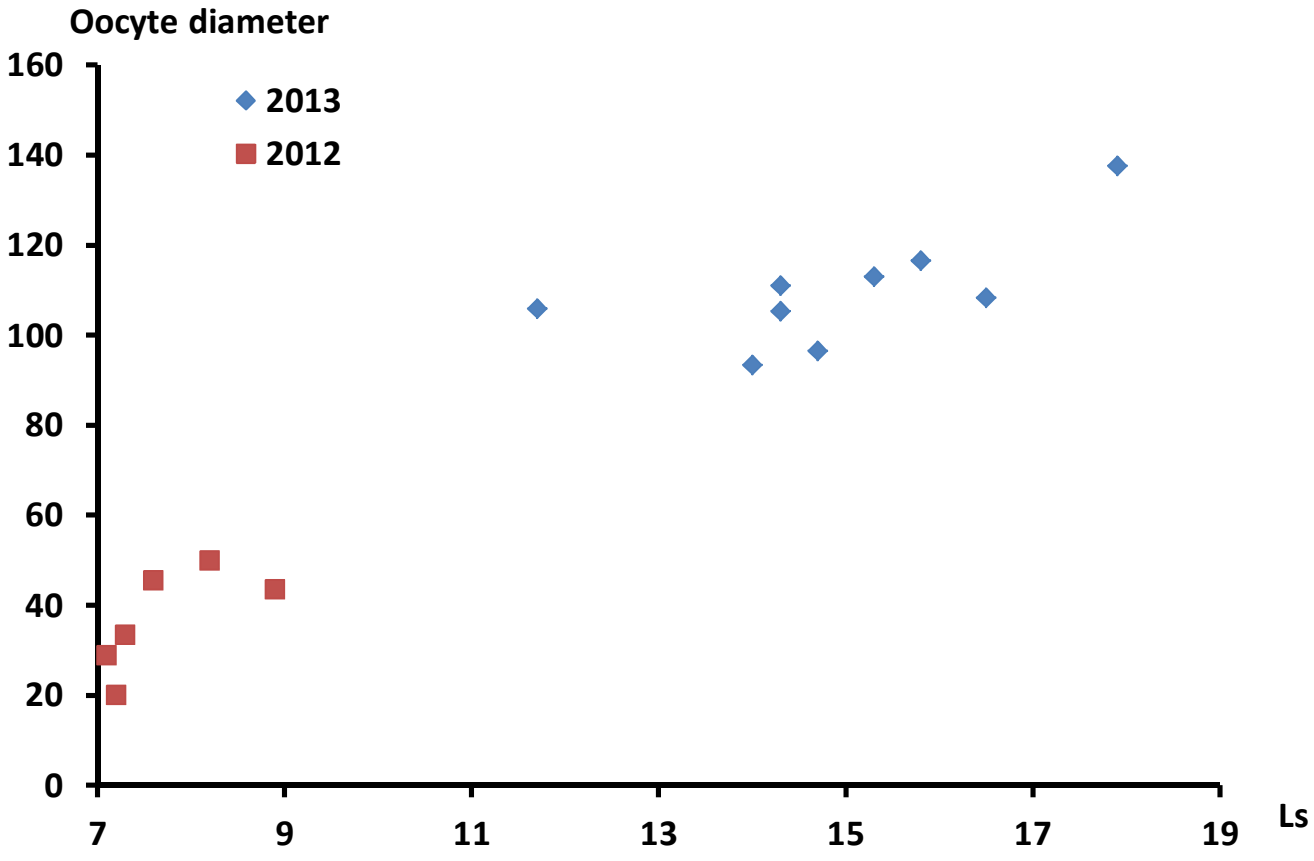


Figure 13: Oocyte diameter (µm) plotted against length (Ls in cm)

3.2.2 Males

Histological observation of the testes showed numerous spermatogonia, with many figures of mitosis. This indicates that the two-year old males are at a spermatogonial stage with active gonial division. The most advanced stages showed formation of cysts (for example. see Photo 14: male n° 25). As compared to one-year old males (see 2nd Report), the testes of two-year old males are larger, but still at the spermatogonia proliferation stage.

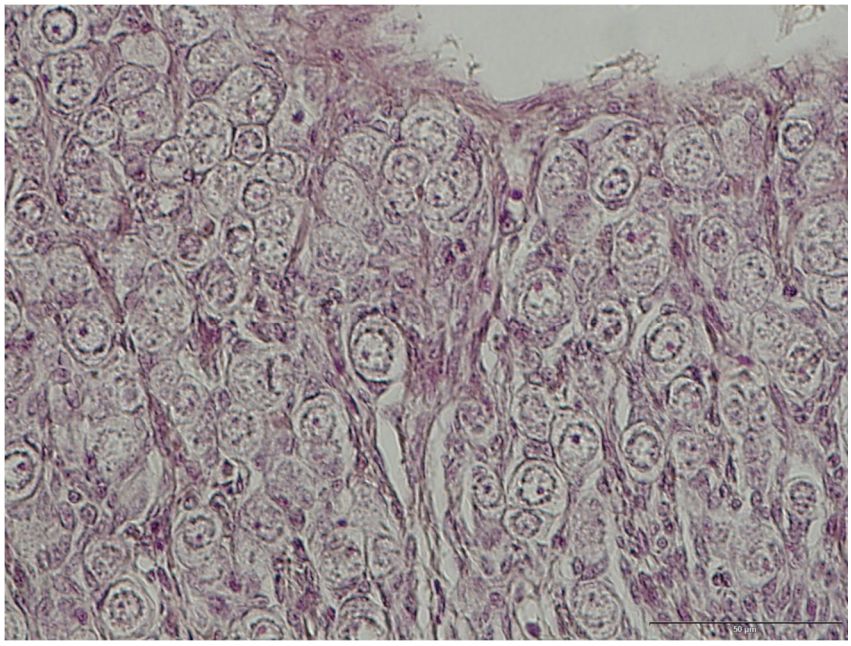


Figure 14: Histological section of testis from two-year old male *Alosa alosa* (Male n° 25; x 40) - Numerous spermatogonia organized in cysts, with many mitoses are observed.

3.3 Biometric parameters of two year old females and males

There was no significant difference between two-year old females and males (Table II) for the body length (females: 14.9 ± 0.6 cm (SEM) ; males: 15.4 ± 0.6 cm; *P*=0.90) nor for the body weight (females: 47.2 ± 5.3 g; males: 50.6 ± 5.6 g; *P*=0.90). These parameters reflect the large body growth of the two year-old fish, as compared to one-year old fish (see 2nd Report) with a mean 2 fold increase in body length and 7 to 8.6 fold increase in body weight for male and female respectively.

The gonadosomatic index (GSI: gonad weight x100/ body weight) ranged between 0.75 and 1.14 in females and between 0.09 and 0.37 in males. Thus, in two-year old shads, the GSI in females (0.94 ± 0.04) was significantly larger than in males (0.17 ± 0.02) ($P < 0.0001$).

The hepatosomatic index (HIS: liver weight x100/ body weight) was 0.67 ± 0.07 in females versus 0.74 ± 0.05 in males ($P= 0.41$).

The digestive tract–somatic index (DTSI: digestive tract weight x100/ body weight) was similar in females (6.99 ± 0.49) and males (6.70 ± 0.60) (P=0.82).

Table II: Sex and biometric parameters of two-year old Alosa alosa, sampled in June 2013 at the Aquarium of La Rochelle. F: female: M: male; GSI: Gonadosomatic index; HSI: hepatosomatic index; DTSI: Digestive tract-somatic index (DTSI).
Gonad sex (ovaries or testis) was determined by visual observation during dissection and further assessed by gonad histology, except for (?) (gonad not retrieved in the tissue sample collected for histology)

Ref fish	Gender	Body lenght (Ls in cm)	Body weight (g)	GSI	HSI	DTSI
25	M	15.3	46.55	0.37	0.60	8.61
26	F	16.5	64.09	0.94	0.41	8.88
27	M	16.5	54.02	0.11	0.57	9.38
28	M	12.2	23.92	0.21	0.96	10.58
29	F	14.3	35.42	1.10	0.65	5.98
30	M	14	37.63	0.11	0.56	5.87
31	F	15.3	54.78	0.86	0.44	8.56
32	F	14.7	43.08	0.91	0.93	8.7
33	M	15.3	44.52	0.09	0.63	6.45
34	F	11.7	21.21	0.99	0.52	6.6
35	F	17.9	74.16	0.98	0.94	4.5
36	M	17.2	77.33	0.19	0.97	6.97
37	F	15.8	52.19	0.75	0.63	6.57
38	*M ?*	*16.4*	*61.45*	*0.11?*	*1.01*	*5.22*
39	F	14	37.01	0.84	0.54	5.89
40	F	14.3	43.06	1.14	0.97	7.24
41	M	14.6	40.75	0.10	0.73	6.01
42	*M ?*	*12.7*	*24.29*	*0.08?*	*0.66*	*4.65*
43	M	17.2	70.43	0.21	0.82	5.56
44	M	16.7	59.95	0.1	0.66	4.33

3.4 Immunoenzymatic assay (Elisa) of 11 Ketotestosterone in adult *Alosa alosa*

11 Keto-testosterone (11-KT) is an androgen characterized in teleosts, and used in some species as a marker of sexually mature males. However, 11-KT was shown to be secreted not only by males but also by females in some species, such as the eel, *Anguilla* species. We investigated the presence of 11-KT in the plasma of adult *Alosa alosa.*

3.4.1 Samples

Plasma were sampled from 5 males and 1 female wild adult *Alosa alosa* caught at Migado's field station, Bruch (June 14, 2012), for inducing reproduction (Table III). Plasma samples were stored at -20°C until assay.

Table III: Biometric parameters and plasma 11-KT levels of wild adults *Alosa alosa* sampled in June 2012

Adult Alosa	Gender	Body length (cm)	Body weight (g)	GSI	11KT (pg/ml)
1	M	53	1050	4.28	197.69
2	M	47.5	685	3.64	396.90
3	F	65	2405	8.52	136.03
4	M	55	1190	6.3	542.55
5	M	49.5	985	2.03	164.81
6	M	46	540	3.7	305.57

3.4.2 Assay of 11-KT in plasma samples from male and female adult *Alosa alosa*

Elisa for 11-KT were performed by SB, using Elisa Kits (Cayman Chemical Company, Ref 582751) previously validated for measuring 11-KT in the eel (*Anguilla Anguilla*) plasma.

Several tests were performed with serial doses of *Alosa* plasma samples. Dose-response curves, parallel to the 11-KT standard, could be obtained between 10 and 0.2 microliters of plasma (Figure 15).

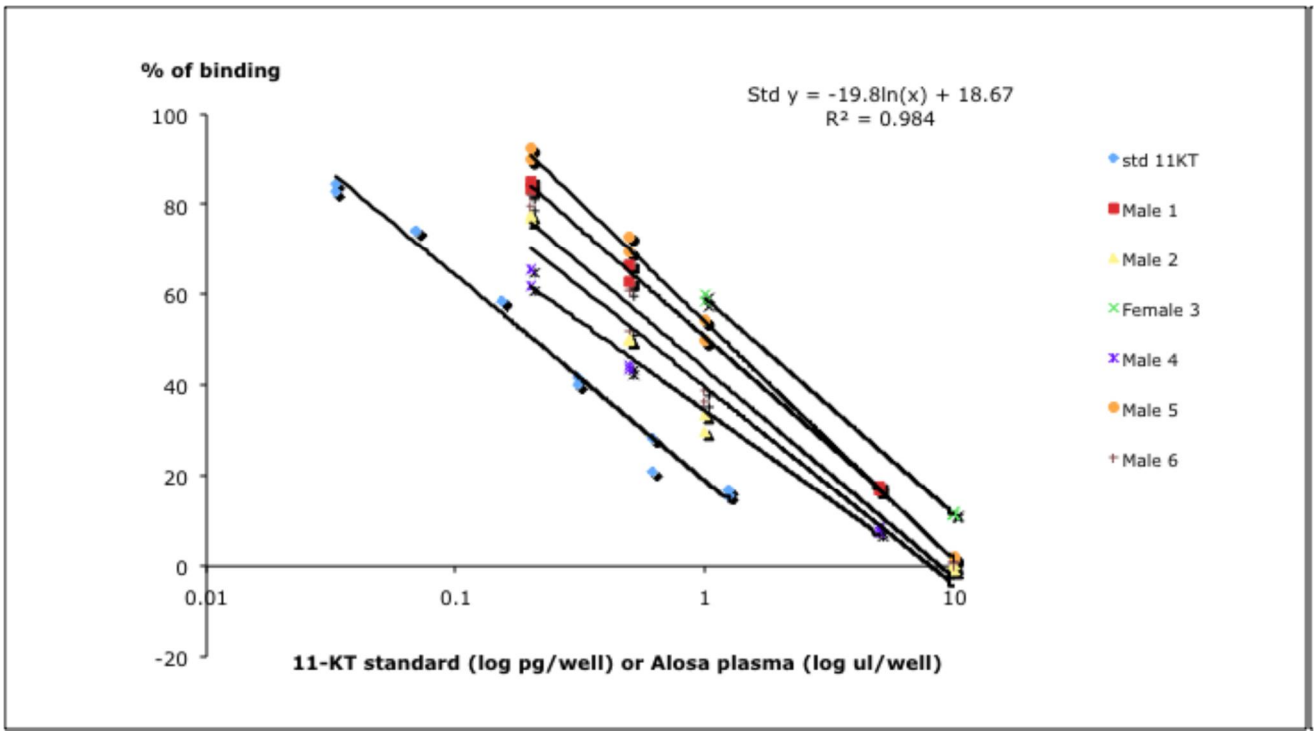


Figure 15: Dose-response curves of 11-KT standard and of serial dilutions of *Alosa alosa* plasma samples in the Elisa for 11-KT

In the 5 males, plasma 11-KT levels ranged between 164 and 542 pg/ml (Table 3) (mean value = 321 $\pm$ 69 pg/ml). The highest level was found in the male with the highest GSI.

11-KT was also well detectable in the plasma of the female, with a value of 136 pg/ml.

3.4.3 Validation of 11-KT assay after plasma extraction

In order to assess whether the detection of 11-KT in female *Alosa* was not an artefact due to crude plasma sample, extraction of the plasma was performed by SB. 100 microliters of plasma were mixed with 200 microliters Elisa Buffer and 3 ml Ether and then frozen in liquid nitrogen (12 sec). The Ether phase was collected, evaporated at 37 °C. Samples were recovered in 200 microliters of Elisa buffer. 11-KT could still be detected by Elisa in extracted plasma samples of both male and female *Alosa alosa*.

These results indicate that 11-KT is produced by both mature males and females in *Alosa alosa* and thus that this androgen is not specific of the males. Such a production by both males and females is also the case in some other teleosts, such as the eel, and other fish such as the sturgeon.

3.4.4 Preliminary Elisa tests for other steroids

Multiple preliminary tests were performed by SB on unextracted and extracted *Alosa alosa* plasma samples for immunoassay of estradiol. However, we found that the kits available at that period (Labor Diagnostica Nord GmbH, Ref FR E-2000) were unsuitable for estradiol assays in fish plasma, due to artefactual crossreactions. Further tests were performed using unextracted and extracted eel plasma, and new kits were tested and compared. Finally the kit Nova Tec, Ref DNOV003 was selected, based on results in the eel.

For Testosterone, we have selected and validated the kit Eurobio Ref EUDNOV002, based on eel plasma samples.

These estradiol and testosterone Elisas are now ready for validation in *Alosa alosa*. We need more plasma samples from wild adults *Alosa alosa* in order to perform these validations.

3.5 Samplings carried out in 2014

Samplings took place the 16 and 17 June 2014.

Twenty two shads were sampled after euthanasia in a concentrated bath of eugenol.

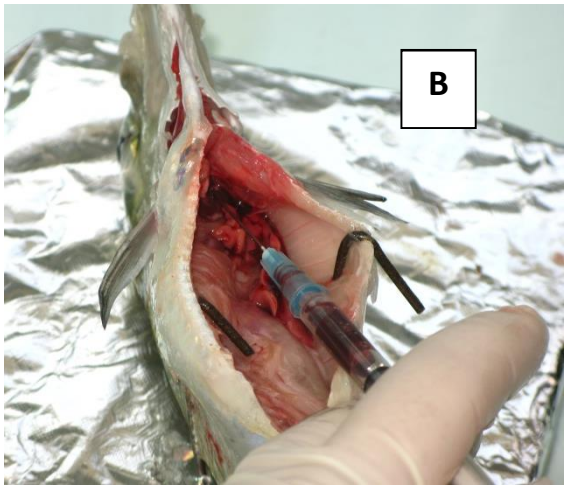
One fish (n°47) was the last survivor of the transfer to freshwater performed during the spring. This male fish had the smallest size, likely reflecting high stress and arrest of feeding. All the other fish were from the batch maintained in seawater.

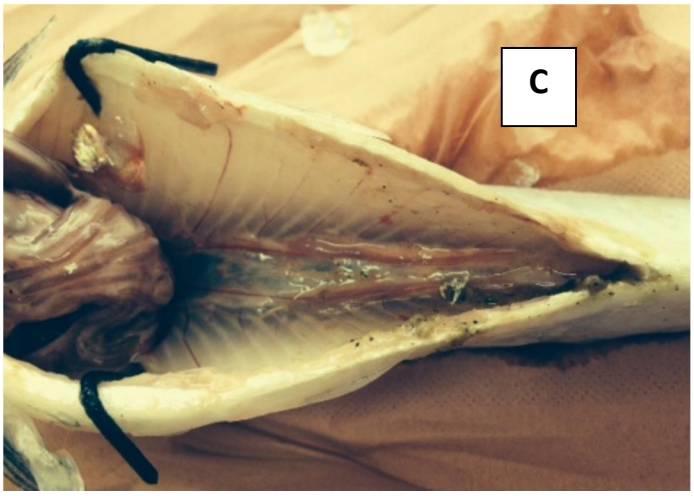
The same protocol was followed as for the previous series of sampling. Fish were individually anesthetized with clove oil. The following external biometric measurements were performed: body length, body weight, body height (at the level of the pectoral fin). After a ventral incision, blood was punctered from the heart using heparinized syringes. Plasma collected after centrifugation was kept frozen at -20°C.

Gonads were easily observable by eye in three-year old fishes (Figures 16), so that they could be dissected out, weighed (for the determination of gonadosomatic index, GSI = % gonad weight/body weight). Gonad was fixed in freshly made Bouin's solution for histological analysis.

The liver and digestive tract were also dissected and weighed for the determination of the hepatosomatic index (HSO) and digestive tract-somatic index (DTSI), respectively.

As such fishes are very unique and precious, additional tissues were sampled, out of the scope of the present project, in order to anticipate possible future molecular investigations: brain and pituitary were dissected under Optika binocular and stored in RNAlater solution (Ambion) at -20 °C. Gonad, gill and liver were also sampled and stored in RNAlater. Some kidney samples, with visible pathological nodules, were fixed for histology.





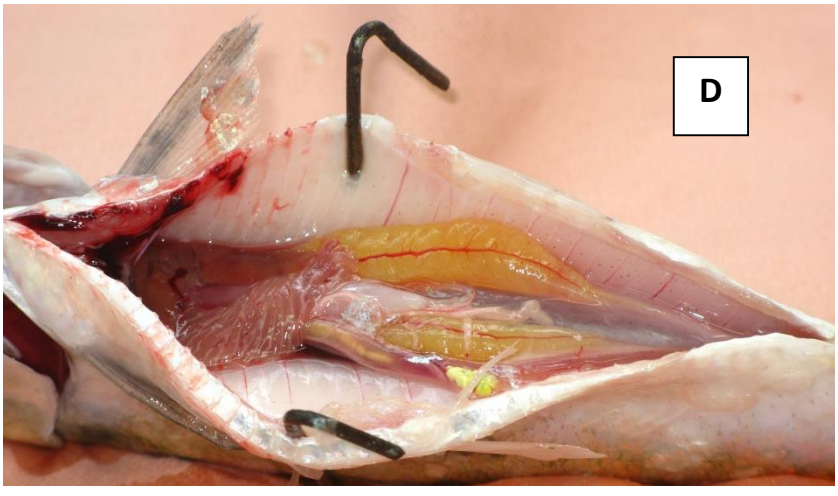


Figure 16: Length measurement, A – blood sample, B – testis, C – ovary, D

During the dissection, ovaries (larger size and orange colour) could be distinguished from testis (smaller size and white colour). Histological analyses to be performed during the next reporting period will allow to assess these observations.

Based on these preliminary observations (to be confirmed), among the 22 fish sampled, 13 would be females and 7 males, with a doubt for two individuals (to be assessed by histology). They would not differ by their body length (21.0 ± 0.6 cm for females *versus* 18.1 ± 1.3 for males) nor by their body weight (129.5 ± 13.9 g for females *versus* 93.0 ± 22.5 g for males).

Females had a significantly higher their gonadosomatic index than males (GSI 0.99 ± 0.08 % in females *versus* 0.23 ± 0.08 % in males; *P*=0.0038).

Three-year old females *Alosa alosa* also had a significantly higher hepatosomatic index than males (HSI 1.09 ± 0.14 in females *versus* 0.72 ± 0.12 in males; *P*=0.0323). This may reflect a higher liver metabolism in females possibly related to vitellogenesis.

Action D7

A new batch of 27 shads has been transferred in the aquaria exposition in September 2013.

A new informative panel will be installed in 2015. This panel will group together information concerning three migratory fish presented in this area, shad (*Alosa alosa*), sturgeon (*Acipenser sturio*) and eel (*Anguilla Anguilla*). An illustration of the project is presented in Figure 17.

Figure 17: Project of the information panel

Technical and management meeting

A half day Life+ project meeting was held at Aquarium La Rochelle on 16 June 2014, with Borea Team and Pierre Morinière from Aquarium La Rochelle. Members of Borea Team presented results of the histological analysis performed on the 2013 samples. Discussions focused also on the schedule for the next season.