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D6.6 Sampling procedures for physiological data

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Executive Summary

Objectives

Commercial cages in seabass aquaculture can hold up to tens of thousands of fish. Samplings for the estimation of the performance of the fish in terms of growth, health and welfare are commonly performed in such cages. It is therefore important to evaluate optimum sampling methods in terms of both randomization and reliable estimation of the traits of interest, as well in terms of welfare.

The first issue to decide is the size of the sample which for common morphometrics, such as weight, and physiological indicators like cortisol and glucose, in a cage population of 10,000 seabass ranges between 5-100 fish in order to provide the required accuracy. Sample size is however subjected to practical (time and costs) and ethical (animal welfare) constraints, encouraging thus researchers, under the concept of the 3Rs (reduce, refine, replace), to seek optimization of sample size and experimental resources according to the goals of the study under consideration.

In terms of welfare, samplings should cause the least possible disturbance to the fish and secure that good welfare conditions are achieved. Furthermore, and in some cases of high importance, the sampling method displayed should cause the minimum possible disturbance to fish so that the physiological traits under study will not be influenced or masked by the sampling itself.

The present study aimed at evaluating different sampling methods from seabass cages in terms of representative sampling for the physiological status of the fish.

Rationale:

Two population of European seabass (*Dicentrarchus labrax*) were used from the HCMR pilot scale farm in north-west Crete. The two populations differed in weight (and age), being 534±145 g the for the large and 168±38 g for the small fish. Both populations were reared in cylindrical cages of the same diameter but different depth. Specifically, large fish were reared in a cage of 8 m depth, while small in 6 m.

Three different sampling methods were evaluated.

- **Hook**; where a set of 12 baited hooks was inserted in the cage and immediately drawn once fish were caught,
- **Net**; where fish were caught after restricting them by the drop of a net attached to the net of the cages,
- **Lift**; where fish were caught after lifting the bottom of the cages and restricting them.

In all sampling methods, fish after capture were immediately anaesthetized, the weight and length of fish were measured, and blood was collected. Three samplings took place for each different sampling method during December 2017 – February 2018.

The physiological parameters estimated were: plasma cortisol concentration, glucose, lactate and haematocrit. Additional immunological parameters were: the serum lysozyme and complement activity, the serum antiprotease and myeloperoxidase activity, the serum nitric oxide and the serum ceruloplasmin activity.

Statistical analysis was performed using the SigmaStat 3.1 statistical package.

Main Results:

Out of the three sampling methods tested only net and lift were successful in all trials and in both populations. Hook was only successful in the first trial, since in the next two trials fish avoided to bite the hook.

The time needed from the initiation of the sampling effort until fish were placed in the anaesthetic varied between sampling methods. In both populations hook was the fastest, net was the second fastest, and lift was the slowest method.

For the Large fish, no differences were observed between the sampling methods in the weight and length of fish. Cortisol, on the other hand, was influenced by the sampling method, with low values observed when hook was compared to net and lift in the first sampling. Net and Lift showed no consistent pattern of differences between them. Glucose was affected by the sampling method, being lower in the hook while higher levels were observed in lift. Differences in lactate existed only between hook and lift being higher in the latter. Finally, no consisted pattern was observed in haematocrit.

For the Small fish, sampling using the hook seemed to collect fish with higher weight, but not length than with net and lift. No differences were observed between net and lift. Cortisol levels, lactate and haematocrit were limited influenced by the sampling method. In glucose lower levels were observed in hook compared to net and lift.

When the large and the small populations were compared in terms of cortisol, it was observed that large fish tend to show lower cortisol levels than small fish.

For the Immunological parameters, the lysozyme activity was significantly reduced in large fish sampled with lift or net compared to hook. Small fish were not affected by the sampling method and lysozyme activity remained unchanged. The anti-protease activity for large fish was significantly lower in net sampling compared to hook while myeloperoxidase activity was not affected by any of the sampling method. On the contrary, the nitric oxide concentration in the sera of the fish was significantly increased in large fish compared to small in lift.

In conclusion, all methods tended to sample fish in a representative way. In terms of welfare, hook was the most rapid and less stressful method as suggested by the lower cortisol and glucose circulating levels. However, hook was not an effective sampling method since it was successful only in first trial. No consistent differences in physiological data were observed between net and lift in both populations, although in large fish a tendency for more fish with low cortisol levels in net compared to lift methods suggests that net might be a milder stressor.

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Sampling procedures in cages for physiological data

1. Introduction and objectives

Commercial cages in seabass aquaculture can hold up to tens of thousands of fish. Samplings for the estimation of the performance of the fish in terms of growth, health and welfare are commonly performed in such cages. It is therefore important to evaluate optimum sampling methods in terms of both randomization and reliable estimation of the traits of interest, as well in terms of welfare.

Firstly, a crucial point is to estimate the minimum number of animals required to get a representative sample of the population or detect an effect if it exists. The latter case is called Type II error (β), which in statistical terms is the retaining of a false hypothesis as opposed to Type I error (α) which is the rejection of a true hypothesis. So, it is desired to reduce type II error in order to be able to detect possible differences between treatments and the probability of not performing this type of error is termed *power of the test* (1-β). Conventionally, the minimum acceptable statistical power is set at 80%.

Statistical power largely depends of the effect size (ES) of the treatment; a treatment with large effect size produces more easily detectable differences and requires less sample size for these differences to be detected. Moreover, statistical power depends on the background variation, which is the variation between experimental units (s^2), as well as on the sample size for each treatment group (n). Finally, it is depended on the level of the probability of Type I error (a), which however is usually set at 5%. For most types of analysis power of the test is:

$$(\mathbf{1}-\boldsymbol{\beta}) \propto \frac{\boldsymbol{ESa}\sqrt{\boldsymbol{n}}}{\boldsymbol{s}}$$

Therefore, the appropriate sample size for a known power of the test is:

$$\sqrt{\boldsymbol{n}} \propto \frac{\boldsymbol{s}(\mathbf{1}-\boldsymbol{\beta})}{\boldsymbol{aES}}$$

In order to be able to calculate the sample size it is important to know the power of the study, the background variation and the ES. Out of these, power of the study is commonly set by the researcher, whereas background variation can be retrieved from pilot studies or respective literature, and finally ES can be estimated by Cohen's d, using the following equations:

$$\boldsymbol{d} = (\bar{\mathbf{x}}_{\mathbf{1}} - \bar{\mathbf{x}}_{\mathbf{2}})/\boldsymbol{s}_{\boldsymbol{pooled}}$$

$$\boldsymbol{s}_{\boldsymbol{pooled}} = \sqrt{\frac{(\boldsymbol{n}_{\mathbf{1}}-\mathbf{1})\boldsymbol{s}_{\mathbf{1}}^{\mathbf{2}} + (\boldsymbol{n}_{\mathbf{2}}-\mathbf{1})\boldsymbol{s}_{\mathbf{2}}^{\mathbf{2}}}{(\boldsymbol{n}_{\mathbf{1}}+\boldsymbol{n}_{\mathbf{2}}-\mathbf{2})}}$$

The calculated sample size required to get a power of 80% for common morphometrics, such as weight, and physiological indicators like cortisol and glucose, in a cage population of 10,000 seabass ranges between 5-100 fish. In extreme cases, for instance in cortisol which can show high variation, samples sizes as high as ~500 fish have been calculated when using highly

dispersed data as the background variation. Sample size is however subjected to practical (time and costs) and ethical (animal welfare) constrains. This encourages researchers, especially under the concept of the 3Rs (reduce, refine, replace), to seek optimization of sample size and experimental resources according to the goals of the study under consideration. In particular, under the 3Rs concept in animal welfare, the number of animals used for experimentation should be reduced when possible, or otherwise use of large sample sizes should be justified when applying for experimental protocol approval, depending also on the severity of the procedure and the necessity and goals of the experiment.

The second important aspect when considering cage sampling, is that the method used should ideally produce the minimum discomfort to sampled fish not affecting their physiological status. In terms of welfare, samplings should cause the least possible disturbance to the fish and secure that good welfare conditions are achieved. For that reason, it is important to study the possible effects that different sampling methods can exert to the fish. Apart from welfare aspects, the sampling method displayed should cause the minimum possible disturbance to fish so that the physiological traits under study will not be influenced by the sampling itself. Most fish species respond to physical disturbance by evoking a stress response; seabass especially is very susceptible to common handling processes responding with high cortisol circulating concentrations (Ellis et al., 2012; Fanouraki et al., 2011). The cortisol stress response is usually observed with a range of minutes after the stressful event (Flik et al., 2006; Rottlant et al., 2003), and the magnitude of the response can depend on the intensity and duration of the stressor (Fatira et al., 2014). Specifically, it has been shown that sampling fish from cages by lifting the bottom of the net leads to duration-dependent increasing in cortisol concentration (unpublished data; Fig 1).

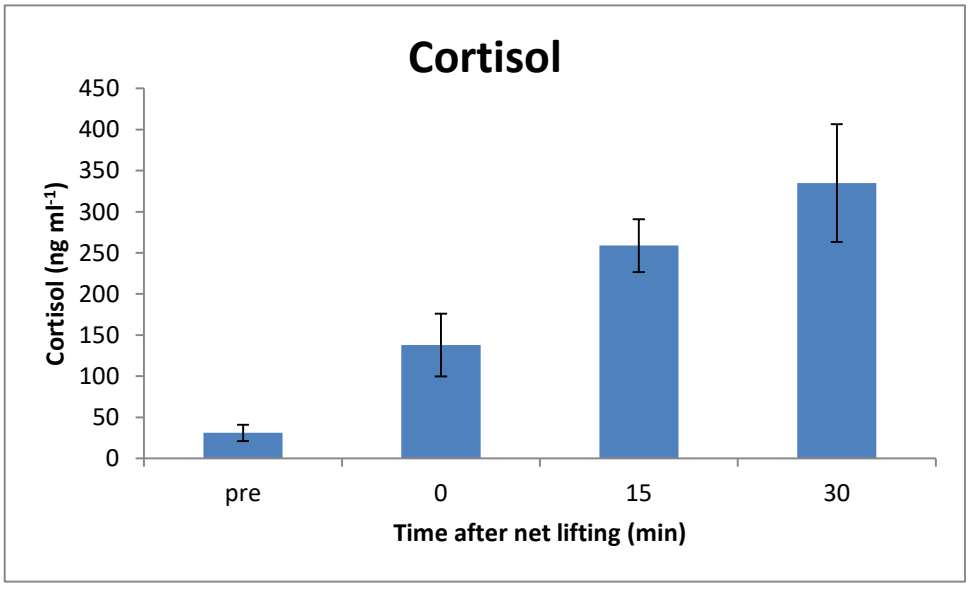

Fig. 1. Mean plasma cortisol levels from seabass sampled prior (pre), at the time (0) and at 15 and 30 minutes after the lift of the bottom of the net (Samaras et al., unpublished data).

Therefore, it is obvious that although a massive number of fish should ideally be sampled from a cage holding tens of thousands of fish, in practice this would contradict ethical (welfare) and practical (change in physiological parameters) aspects. In detail, sampling of such a number of fish from sea cages needs much time that will result is poor welfare for the fish, as well as

affect the results of physiological data, such as cortisol, glucose and lactate due to the stress that will be induced to the fish. Therefore, it is of major importance that the sampling is as rapid and as less stressing as possible. For that reason, in the present study a number of 15 fish per sampling was used as a compromise between the actual calculated sampling size based on previous experiments and the practically feasible number of sampled fish due to physiological (*i.e.* increased time to collect fish and blood samples will affect physiological data) and logistic reasons (*i.e.* simulating the practical number of personnel needed to sample fish in an aquaculture unit).

In this context, the present study aimed at evaluating different sampling methods from seabass cages in terms of representative sampling for the physiological status of the fish. To do so, morphometric and physiological parameters were quantified for two population of seabass sampled from sea cages using three sampling methods. Briefly, the methods used were (i) sampling by hook and line; (ii) using an external net to rapidly restrict part of the population of the cage before sampling, and (iii) lifting the bottom of the cage-net to restrict all fish before sampling.

2. Methods

2.1 Site and rearing

Two population of European seabass (*Dicentrarchus labrax*) reared in the pilot scale farm of Hellenic Centre for Marine Research (HCMR) located at Souda Bay in north-west Crete. The two populations tested differed in their weight (and age), being (mean (SD)) 534.85 (145.84) g in the large and 168.45 (38.04) g in the small population. Both populations were reared in the same stocking density (~8 kg/m^3) in cylindrical cages of the same diameter but different depth. Specifically, large fish were reared in a cage of 8 m depth, while small in 6 m.

Fish were offered standard extruded commercial diets (Irida S.A., Greece) of approximately 44% protein and 19% lipids by automated feeders twice daily throughout the rearing period. The provided amount was calculated according to feeding tables for the species.

2.2 Comparison of sampling methods

Three different sampling methods were evaluated. Specifically, these methods were:

1. **Hook**; where a set of 12 baited hooks was inserted in the cage and immediately drawn once fish were caught,
2. **Net**; where fish were caught after restricting them by the drop of a net attached to the net of the cages,
3. **Lift**; where fish were caught after lifting the bottom of the cages and restricting them.

In more details, **hook** consisted of a fishing line equipped with 12 hooks. The fishing line was introduced in the cage extending from one side to the other (Fig. 2). Immediately after placing the hooks in the cages fish were caught and collected. In the first attempt, some fish bit the hooks in matter of seconds and were collected immediately, not waiting for all 12 hooks to be

bitten. The whole procedure from biting to anaesthesia lasted approximately 1 minute (as presented in the results; table 1).

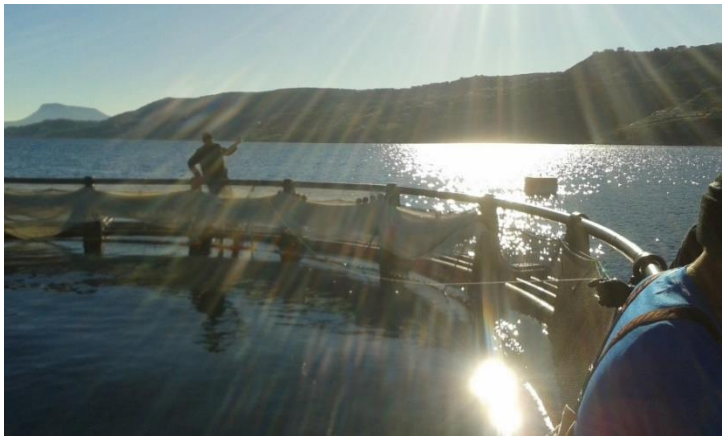

Fig 2. Photograph presenting the hook method. The fishing line is equipped with 12 hooks, which were immediately inserted in the cage from side to side.

The **net** method consisted of sampling fishing by restricting a random subpopulation using a net pre-attached to the net of the cage (Fig. 3). Specifically, one side of the net was tied to one side of the cage (Fig. 3A), while the other part was thrown in the water and pulled in order to create a cavity where the fish were restricted (Fig. 3B). Once this was accomplished fish were immediately collected by a fish net and placed in anesthesia.

Fig 3. Photographs presenting the net method. The left photograph depicts the net prior to insertion in the water, while the right shows how the fish are confided prior to sampling.

Finally, **lift** was accomplished by lifting the net of the cage to confine fish and subsequently sampling them using a fish net. Lift was the most time-consuming method (Table 1) as well as led to a more vigorous confinement of the fish (Fig. 4).

Fig 4. Photographs presenting the lift method. The left photograph shows how the bottom of the cage net is lifted to confine fish and the right depicts the confined fish.

In all sampling methods fish after capture were immediately anaesthetized in ethyl-glycol monophenyl ether (0.2 ml l^{-1}), weight and length measurements were recorded, and blood was immediately collected through the caudal vessel via heparinized syringes and placed in heparinized collection tubes. Finally, blood was centrifuged at 2,000 g for 10 minutes, and stored at -20°C until analyzed for further analysis.

In total three samplings took place for each different sampling method, which are referred to as S1, S2 and S3, respectively. These samplings were performed during December 2017 – February 2018.

2.3 Analytical procedures

2.3.1 Physiological parameters

Plasma cortisol concentration was quantified by a commercial enzyme immunoassay kit (DRG® Cortisol ELISA; Germany) previously evaluated in seabass (Samaras et al., 2016). Glucose and lactate were quantified by commercial colorimetric assays (Biosis, Greece for glucose; Spinreact, Spain for lactate). Finally, haematocrit was measured in capillary tubes after centrifugation in a haematocrit microcentrifuge.

2.3.2 Serum lysozyme activity

Lysozyme was measured using the turbidimetric method described before (Kokou et al., 2012). Briefly, the kinetic of lysis of the membrane of Micrococcus luteus (0.2 mg ml^{-1}) by 10μl of serum was followed at 450 nm for 20 min (Genios Pro, Tecan, Austria). Results are expressed as units/ml of serum.

2.3.3 Serum complement activity

The antibacterial activity of the complement in serum of the fish was determined as described before (Kokou et al., 2012). It was expressed as the percentage of bacterial killing against a luminescent strain of E.coli.

2.3.4 Serum antiprotease activity

The method to determine the anti-protease activity of the serum, which evaluates the ability of the fish immune system to fight parasites, was adapted from Magnadóttir et al. (1999). Briefly, 5 µl of serum or phosphate buffer saline (PBS for negative control) were incubated with 20 µl of standard trypsin solution at 5 mg/ml for 10 min at 22 °C in a round-bottom 96-well microplate. A standard curve was prepared with increasing volumes of trypsin diluted with PBS. Then 60 µl of 1% (w/v) azocasein solution were added and incubation further lasted for 1 h. Then, 100 µl of 10% TCA were added and incubated for 30 additional minutes. The microplate was then centrifuged and 100 µl of the supernatant were transferred to a clean flat-bottom 96 well transparent microplate and 100 µl of 1 N NaOH were added in each well. The OD was then read at 450 nm. Results were expressed as the percentage of trypsin inhibition calculated using the standard curve as a reference (Henry and Fountoulaki, 2014).

2.3.5 Serum myeloperoxidase activity

The myeloperoxidase activity of serum was determined as described before (Kokou et al., 2012) but using 50 µl of the stopping solution (Henry et al., 2015). Briefly, 15 µl of serum were diluted with 135 µl HBSS and 50 µl of the TMB-H2O2 solution were incubated for 2 minutes before 1 N H2SO4 was added to stop the reaction. OD was measured at 450 nm (Genios Pro, Tecan, Austria).

2.3.6 Serum nitric oxide

The nitric oxide concentration in serum was determined as described before using the Griess reaction (Henry et al., 2009).

2.3.7 Serum ceruloplasmin activity

The ceruloplasmin oxidase activity, which is considered to be a marker of the inflammatory response, was measured following the previously described method (Dunier et al., 1995) using 10 µl of serum incubated with 100µl of the 0.1% para-phenylenediamine solution. The kinetic of increase of absorbance was followed at 550 nm for 15 min (Genios Pro, Tecan, Austria) and 1 unit was defined as an increase of OD of 0.001/min (Henry and Fountoulaki, 2014).

2.4 Statistical analysis

Statistical analysis was performed using the SigmaStat 3.1 statistical package (Systat Software, Inc; USA). Before analysis data were checked for meeting the assumption criteria of the respective statistical test. Data on each sampling were analysed separately by one-way ANOVA in S1 (comparing the three sampling methods), and t-test in S2-S3 (comparing Net and Lift). When statistically significant differences were observed in the one-way ANOVA, Tukey's multiple comparisons were performed. The reason for not using a two-way ANOVA to test the factors "method" and "sampling time" simultaneously was due to the fact that (i) in Hook only data for S1 were available, therefore not allowing the use of a two-way ANOVA, and (ii) the main interest of the present study was to examine the effects of different sampling methods in each sampling period and not between different samplings.

3. Results

Out of the three sampling methods tested only net and lift were successful in all trials and in both population. Hook was only successful in the first trial, since in the next two trials fish avoided to bite the hook.

The time needed from the initiation of the sampling effort until fish were placed in the anaesthetic varied between sampling methods (Table 1). In both populations hook was the fastest sampling method. Since, however, only one trial was successful it was not feasible to calculate the average time. Moreover, in both population net was the second fastest method, and lift was the slowest. In the small fish, especially, net was very fast compared to lift.

Table 1. Time from the initiation of the sampling till the fish were immersed in anaesthesia. Time is presented as mean (SD) in minutes. In the case of hook less than 1 minute was needed.

	Large	Small
Hook	~ 1	~ 1
Net	9.67 (4.54)	3.83 (1.04)
Lift	15.83 (1.04)	15.00 (6.54)

3.1 Large fish

No differences were observed between the sampling methods in the weight, length and condition factor of fish (Fig. 5).

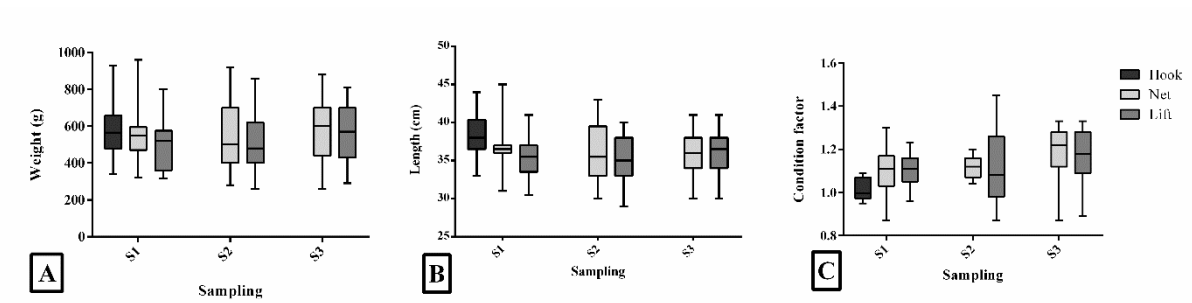

Fig 5. Weight (A), length (B) and condition factor (C) of the large seabass sampled using the 3 different sampling methods in the 3 samplings. The box-plot represents the interquartile range between the 1st and 3rd quartiles, while the horizontal line inside the box-plot represents the median. Whiskers represent the minimum and maximum values. n = 8 in Hook; n = 15 in Net and Lift.

Cortisol, on the other hand, was influenced by the sampling method (Fig. 6A). Specifically, low values were observed when hook was compared to net and lift in the first sampling. Net and lift, however, showed no consistent pattern of differences between them. In details, no statistically significant differences were observed in S1 and S3, while in S2 higher cortisol levels were observed in the lift compared to the net method.

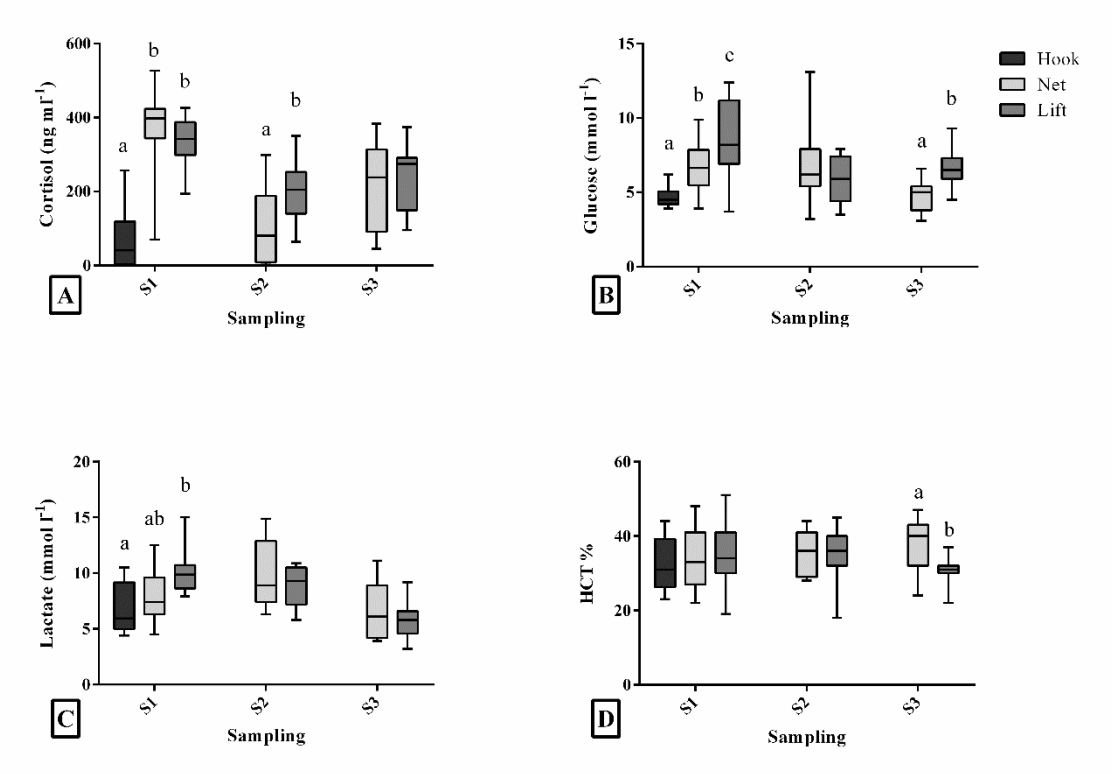

Fig 6. Concentrations of cortisol (A), glucose (B), lactate (C), and HCT% of the large seabass sampled using the 3 different sampling methods in the 3 samplings. The box-plot represents the interquartile range between the 1st and 3rd quartiles, while the horizontal line inside the box-plot represents the median. Whiskers represent the minimum and maximum values. n = 8 in Hook; n = 15 in Net and Lift. Different letters indicate statistically significant differences between sampling methods in each sampling ($P < 0.05$).

Glucose was also affected by the sampling method, being lower in the hook compared to the other methods. Moreover, higher glucose levels were observed in lift when compared to the net in S2 and S3 (Fig. 6B). Differences in lactate existed only between hook and lift in S1, being statistically significant higher in the latter (Fig. 6C). Finally, no consisted pattern was observed in haematocrit, showing a significant difference only between net and lift in S3 (Fig. 6D).

3.2 Small fish

Sampling using the hook method seemed to collect fish with significantly higher weight, but not length nor condition factor, than net and lift (Fig. 7). No differences were observed between net and lift in any sampling period.

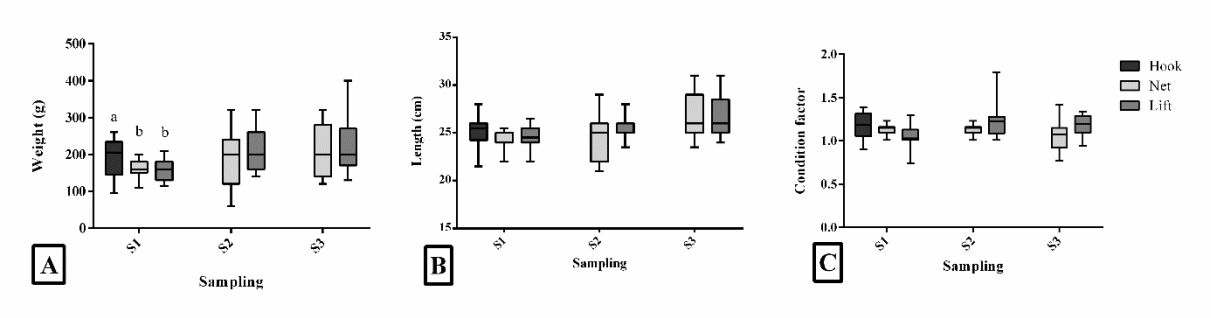

Fig 7. Weight (A), length (B) and condition factor (C) of the small seabass sampled using the 3 different sampling methods in the 3 samplings. The box-plot represents the interquartile range between the 1st and 3rd quartiles, while the horizontal line inside the box-plot represents the median. Whiskers represent the minimum and maximum values. n = 12 in Hook; n = 15 in Net and Lift. Different letters indicate statistically significant differences between sampling methods in each sampling ($P < 0.05$).

Cortisol levels in the small population were not intensively influenced by the sampling method. The only differences that were observed regarded increased levels between lift and the other two methods in S1 and between net and lift in S3 (Fig. 8A). The only difference observed in glucose regarded the significantly lower levels in hook compared to net and lift (Fig. 8B). Lactate showed differences only between net and lift in S2, being lower in the latter (Fig. 8C). Haematocrit differed only between net and lift in S3 (Fig. 8D).

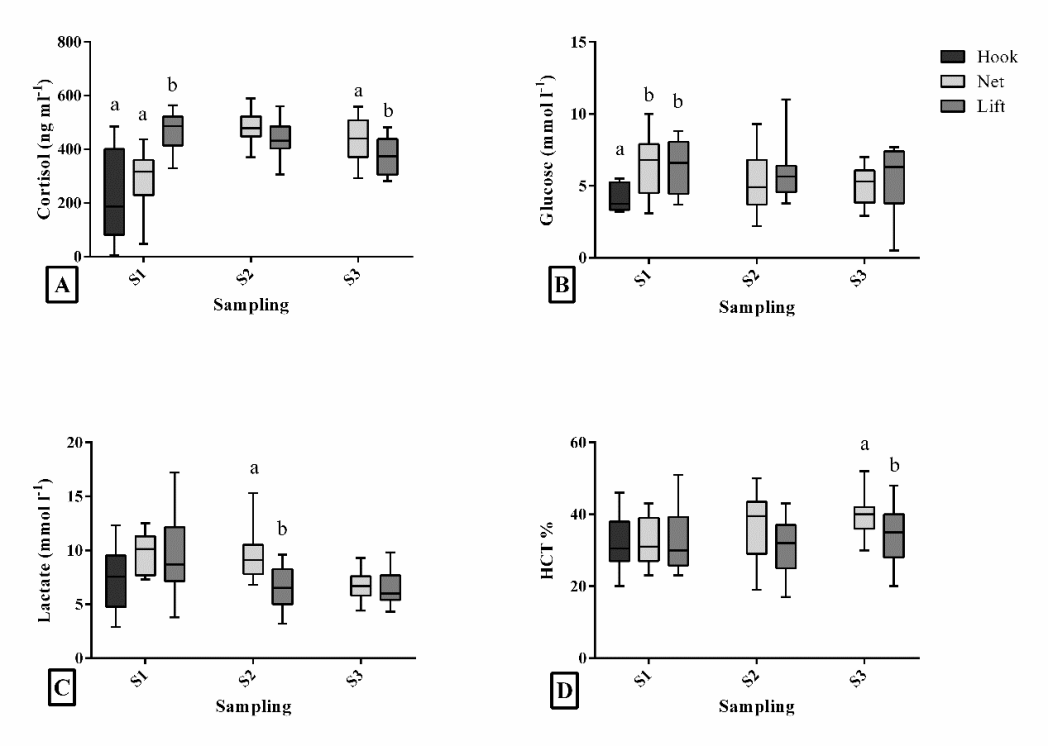

Fig 8. Concentrations of cortisol (A), glucose (B), lactate (C), and HCT% of the small seabass sampled using the 3 different sampling methods in the 3 samplings. The box-plot represents the interquartile range between the 1st and 3rd quartiles, while the horizontal line inside the box-plot represents the median. Whiskers represent the minimum and maximum values. n = 12 in Hook; n = 15 in Net and Lift. Different letters indicate statistically significant differences between sampling methods in each sampling ($P < 0.05$).

3.3 Comparison between large and small fish in cortisol

When the two populations were compared in terms of cortisol (Fig 9), which is one of the main hormones regulating stress responses, it was observed that large fish tend to show lower cortisol levels than small fish (Table 4). Specifically, this was observed in the hook and lift method, and in S1 and S2 of the net method.

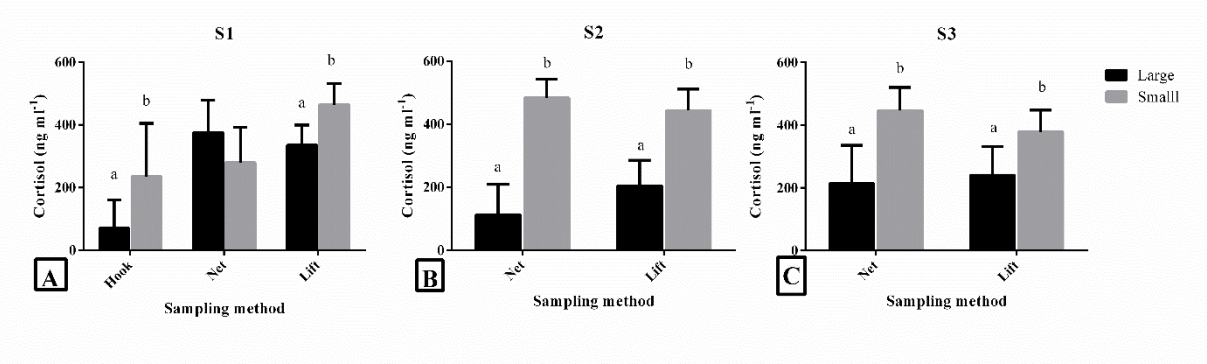

Fig 9. Concentrations of cortisol in large and small fish in S1 (A), S2 (B), S3 (C), sampled using the 3 different sampling methods in the 3 samplings. Different letters indicate statistically significant differences between sampling methods in each sampling ($P < 0.05$).

Table 2. ANOVA (F) table for cortisol between large and small fish.

Sampling	Factor	Df1	Df2	F	p
S1	Method	2	73	36.18	*< 0.0001*
	Population	1	73	7.28	*0.009*
	Interaction	2	73	11.16	*< 0.0001*
S2	Method	1	54	1.61	0.210
	Population	1	54	225.1	*< 0.0001*
	Interaction	1	54	10.50	*0.002*
S3	Method	1	56	0.76	0.387
	Population	1	56	61.55	*< 0.0001*
	Interaction	1	56	3.94	0.052

3.4 Immunological parameters

The figures below presents the results related to the immunological parameters estimated during the trial

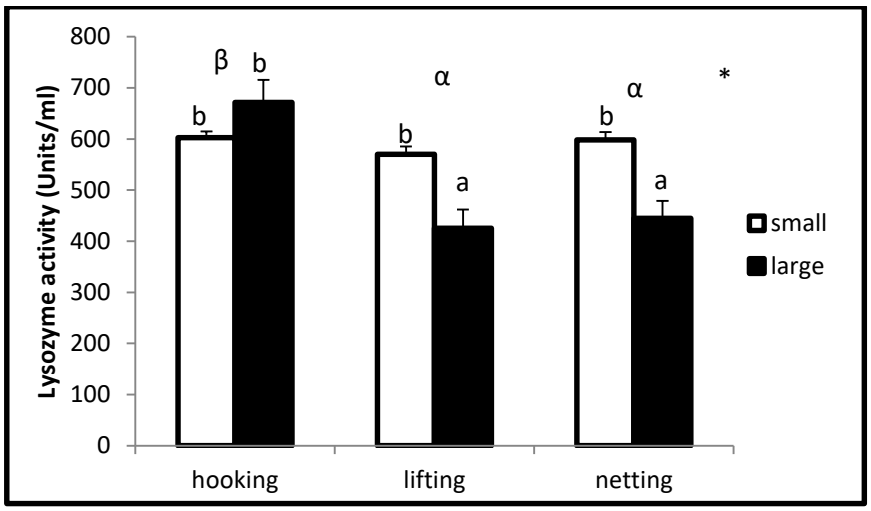

Fig. 10. Lysozyme antibacterial activity in the serum of small (open bars) or large (black bars) fish sampled using different sampling methods from the sea cages (hooking, lifting or netting). Bars represent mean ± S.E.M. Different Latin letters show significant differences between sampling methods in small or large fish as different groups (Kruskal-Wallis, P=5.10-6, Tamhane). Different Greek letters show significant differences between fishing techniques in all fish, small and large together (General Linear Method, P=0.002) and asterisk show significant difference between small and large fish (GLM, P=0.009). n=12-45.

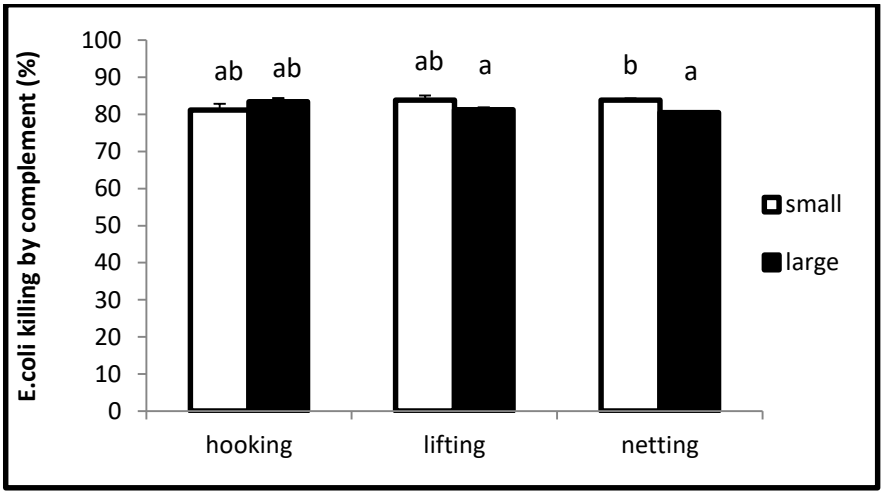

Fig. 11. Complement antibacterial activity in serum of small (open bars) or large (black bars) fish sampled using different sampling methods from the sea cages (hooking, lifting or netting). Bars represent mean ± S.E.M. Different Latin letters show significant differences between sampling methods in small or large fish as different groups (Kruskal-Wallis, P= 0.0002, Tamhane). There were no significant differences between fishing techniques or fish sizes (GLM, P>0.05). n=12-45.

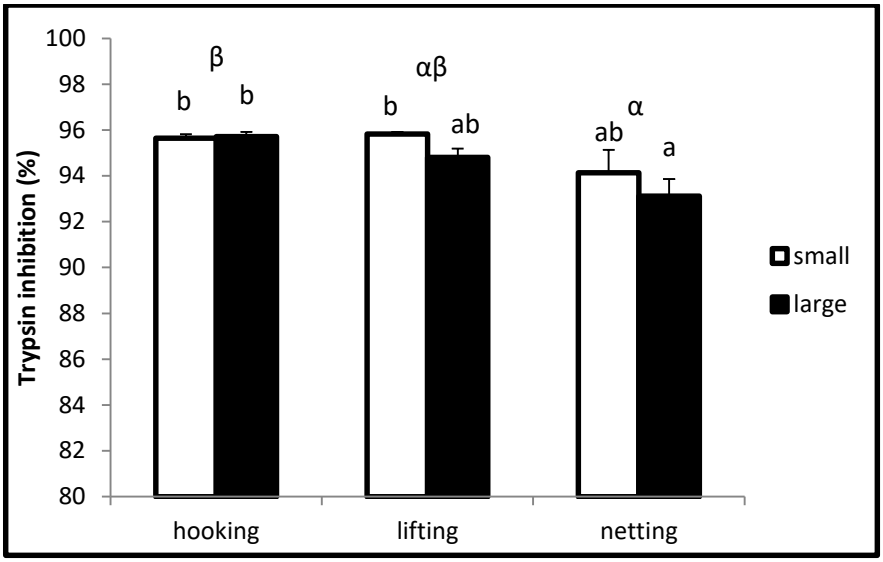

Fig. 12. Trypsin inhibition in the serum of small (open bars) or large (black bars) fish sampled using different sampling methods from the sea cages (hooking, lifting or netting). Bars represent mean ± S.E.M. Different Latin letters show significant differences between sampling techmethodsniques in small or large fish as different groups (Kruskal-Wallis, P= 0.0003, Tamhane). Different Greek letters show significant differences between fishing techniques in all fish small and large together (General Linear Method, P=0.012). There were no significant differences between small and large fish (GLM, P>0.05). n=12-45.

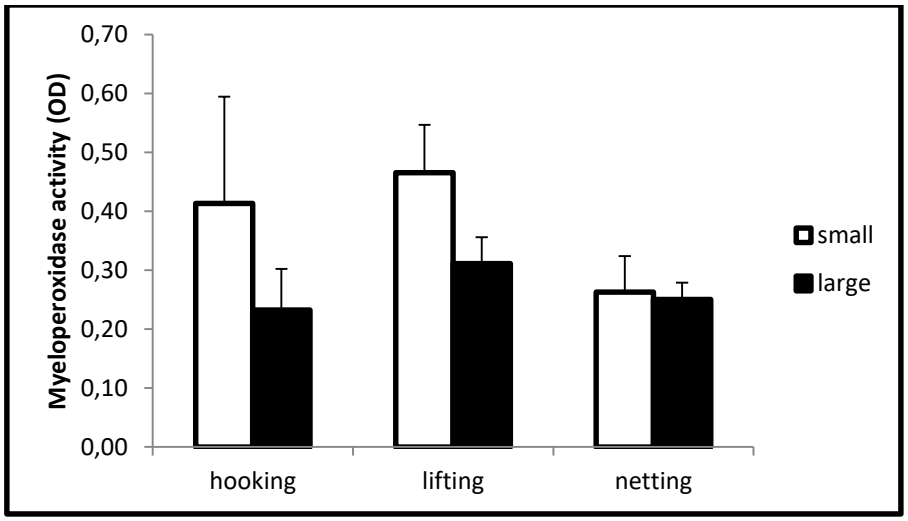

Fig. 13. Myeloperoxidase activity in the serum of small (open bars) or large (black bars) fish sampled using different sampling methods from the sea cages (hooking, lifting or netting). Bars represent mean ± S.E.M. Different Latin letters show significant differences between sampling methods in small or large fish as different groups (Kruskal-Wallis, P>0.05, Tamhane). There were no significant differences between fishing techniques or fish sizes (GLM, P>0.05). n=12-45.

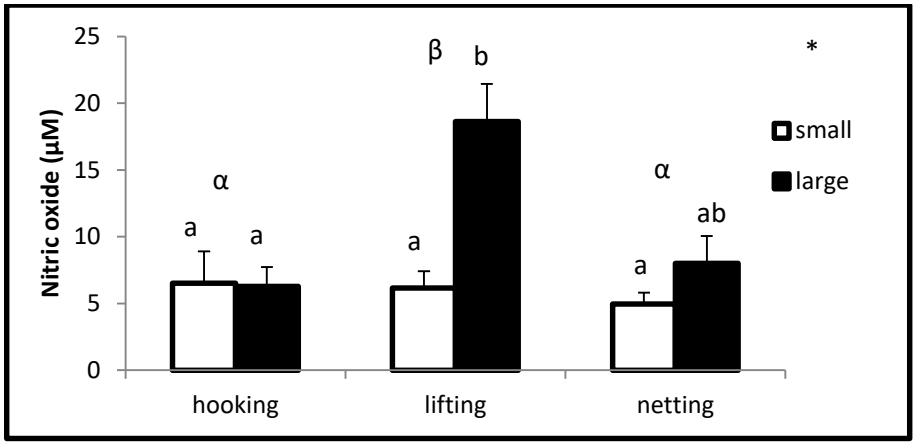

Fig. 14. Nitric oxide concentration in the serum of small (open bars) or large (black bars) fish sampled using different sampling methods from the sea cages (hooking, lifting or netting). Bars represent mean ± S.E.M. Different Latin letters show significant differences between sampling methods in small or large fish as different groups (Kruskal-Wallis, P= 0.0002, Tamhane). Different Greek letters show significant differences between fishing techniques in all fish small and large together (General Linear Method, P=0.004) and asterisk show significant difference between small and large fish (GLM, P=0.014). n=12-45.

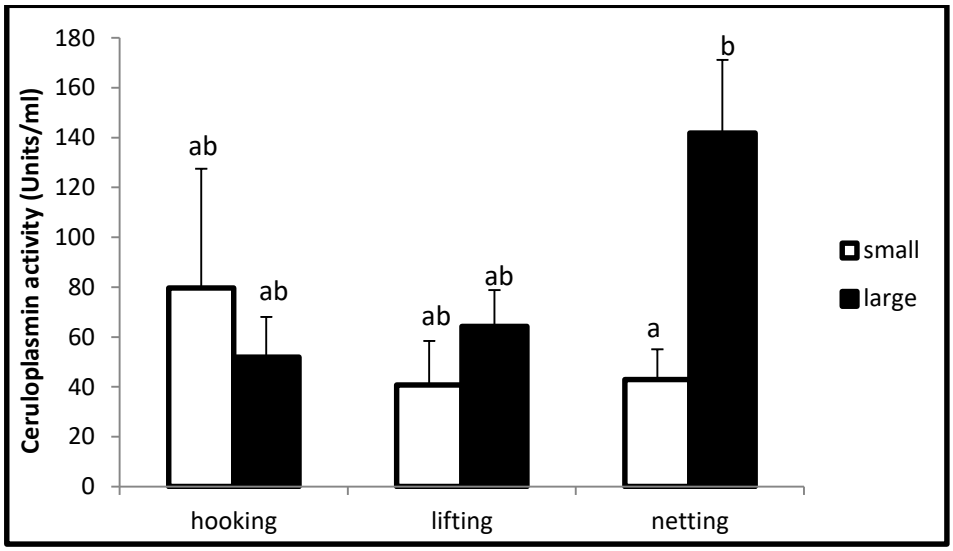

Fig. 15. Ceruloplasmin activity in the serum of small (open bars) or large (black bars) fish sampled using different sampling methods from the sea cages (hooking, lifting or netting). Bars represent mean ± S.E.M. Different Latin letters show significant differences between sampling methods in small or large fish as different groups (Kruskal-Wallis, P=0.03, Tamhane). There were no significant differences between fishing techniques or fish sizes (GLM, P>0.05). n=12-45.

The Pearson correlation test was performed between all the immune parameters determined in the present study for all fish, for small fish alone and for large fish alone and results are presented in Table 5. The ceruloplasmin was slightly positively correlated to the myeloperoxidase activity ($P = 0.04$) mainly because of small fish ($P = 0.0031$ in small fish; $P > 0.05$ in large fish) and to nitric oxide ($P = 0.073$; $P = 0.002$ in small, $P = 0.047$ in large). The complement activity was strongly positively correlated to both the lysozyme ($P = 0.0001$, $P > 0.05$ in small; $P = 2.10^{-8}$ in large) and the anti-protease activity ($P = 0.003$; $P > 0.05$ in small; P

= 0.00006 in large fish) mainly because of large fish. The lysozyme activity was strongly negatively correlated to the nitric oxide ($P = 8.10^{-7}$; $P > 0.05$ in small fish; $P = 0.003$ in large fish) and to a lesser degree to the ceruloplasmin activity ($P = 0.003$) but, like the complement antibacterial activity, it was positively correlated to the anti-protease activity ($P = 0.0032$) due to results in large fish.

Table 5. Pearson's correlation between all immune parameters. "+" denotes a positive correlation; "-"denotes a negative correlation. 1 symbol represent a significance at P<0.01; 2 symbols at P< 0.05; 3 symbols at P<0.001 and 4 symbols at P<0.0001. In brackets are shown the correlation concerning only the small or only the large fish (small/large).

	Complement	APA	MPO	NO	Ceruloplasmin
Lysozyme	++++ (0/++++)	+++(0/++++)	0(-/0)	----(0/----)	---(0/--)
complement		++++(0/++++)	0(0/0)	0(0/0)	0(0/--)
APA			0(0/0)	0(0/0)	0(0/0)
MPO				+(+++/++)	++(+++/0)
NO					0(++/0)

The lysozyme activity was significantly reduced in fish sampled by lifting the net or netting them compared to fish fished out of the cage especially so concerning the larger fish (Fig. 10). Small fish were not affected by the fishing technique and lysozyme activity remained unchanged. The anti-protease activity was also significantly lower in fish netted compared to fish fished out of the cages, especially so in large fish (Fig. 12). Myeloperoxidase activity was not significantly affected by any of the fishing techniques, although it tended to be reduced in larger fish compared to small ones (Fig. 13). This difference was not present in the netted fish.

On the contrary, the nitric oxide concentration in the sera of the fish was significantly increased in fish large fish compared to the small fish in the cage which was lifted to sample the fish (Fig 14). The ceruloplasmin activity was increased in large fish compare to small netted fish suggesting an inflammation process in these fish (Fig, 15). However, standard error was relatively high denoting strong inter-individuals differences and a high variability of the ceruloplasmin activity in fish sampled the same way with values varying between 0 and 760.

4. Discussion

Sampling fish from cages faces three major challenges. The first is to collect a representative sample of the population, both in terms of individual characteristics (for instance netting usually captures larger fish first), as well as in terms of sample size. The second is to use a method that promotes welfare and minimizes stress that can negatively influence the results. Finally, the third challenge is about using a sampling method that manages to cover the above-mentioned issues in fish of different sizes.

For these reasons, the present study aimed at comparing three different methods for the sampling fish from sea cages in two seabass populations of different size. The methods that were used were the common practice of lifting the bottom of the cage to confine fish; a newly developed method of using a net to confine fish before sampling; and finally catching fish using baited hooks. All three methods were tested in a population of commercially-sized fish (~ 500 g; termed as "large") and a population of smaller (~ 200 g; termed as "small").

Results showed that net and lift methods were consistently successful in sampling fish, whereas hook was only successful in the first sampling. Specifically, during the second and third sampling no fish bite the hooks in both populations. Such a learned hook avoidance behavior is not universal among fishes and for many species it is a viable sampling method. However, it has been reported for species such as rainbow trout, pike and carp (Askey *et al.*, 2006). In many cases it seems to be a trait that individuals pick up fast, often within a single hook encounter which is in agreement with the findings of this study. Although the mechanisms underlying hook avoidance have not been fully disentangled, recent studies suggest that there is no social element to learning this behaviour which is rather based on prior experience regarding the morphology of hooks (Louison *et al*., 2019).

No significant differences in the morphometric features of the fish sampled with the different methods were observed. The fact that no consistent differences existed among the sampling methods indicates that there was an equal representation of the population using each of these methods. There was only a statistically significant difference with heavier, but not longer, (neither with larger condition factor), fish sampled using the hook method in the population of small fish. This might indicate a size-dependent difference between large and small fish, where in the latter heavier fish tend to be the first to approach food due to social interactions and hierarchies (Andrew et al., 2002).

Physiological parameters showed to be influenced by the different sampling methods, but not in a consistent pattern. Specifically, in the first sampling where fish were caught using the hook method, lower plasma cortisol concentrations were observed in the large fish sampled by the hook than the other two methods, while in small fish sampling by hook and net resulted in lower cortisol than the lift method. As the sampling time was apx 1 min, this was not enough period to elicit a response in the parameters that were tested. Given the fact that cortisol concentration starts to increase few minutes after stress (Flik et al., 2006; Rottlant et al., 2003), lower levels were expected when using the hook method, which was observed in the population of large fish (71.4 ± 89.0 ng ml^{-1}), but not in small (235.7 ± 169.1 ng ml^{-1}). This fact could probably suggest either that small fish population was stressed due to an unknown, uncontrolled factor or that these fish were more susceptible to the same stimuli (i.e. hook) than the large fish. The size of the fish should not *per se* be the driving force between the differences in cortisol, since no differences in resting and post-stress cortisol levels have been observed between seabass of the same size order (127.0g Vs 351.5g; Fanouraki 2010). It is important to notice however that in large fish a part of the fish is expected to be sexually mature, and especially at the time of spawning. In general, reduced cortisol levels are observed during the spawning period, which however coincides with the low winter sea temperatures and it is not clear whether this is due to the reproductive state or temperature (Planas et al., 1990; Pascoli 2011; Samaras et al., 2016). In support of a strong temperature effect is the fact that low cortisol levels have been observed in sexually immature seabass reared at low temperature (Samaras et al., submitted).

In general, no consistent differences were observed between the net and lift methods in neither populations. Specifically, in large fish significantly higher levels of cortisol where observed in fish sampled using the lift method than the net in S2. On the other hand, the same difference was observed in small fish in S1, while the opposite was true (*i.e.* higher levels in net than lift) in S3. Therefore, it is hard to conclude that a consistent effect of the sampling method exists between the net and lift methods. However, when examining the population of large fish, a

very high variation in cortisol is observed when using the net method (Fig 5A), indicating that some fish do not show a cortisol response while other fish do. It seems therefore that net is a somehow milder method to sample fish, possibly due to the fact that it is faster than the lift method. The difference in the duration of the procedures (9.7 Vs 15.8 minutes in net and lift, respectively) could also have slightly affected cortisol outcome due to the time-dependence nature of the response, which reaches max levels between 0.5 and 1 hour post-stress (Fanouraki et al., 2011). In small fish, however, such differences were not evident (even though net was even faster), which accompanied with the high cortisol levels in the hook method, suggests that these fish were more susceptible to stress. This is also supported by the fact that the population of small fish showed higher cortisol levels than the large fish in all samplings.

Glucose is another indicator commonly used to assess the welfare of fish. In both populations lower glucose levels were observed in hooked individuals compared to the other sampling methods. This further suggests that hook is the less stressing method due to its rapidness, especially because glucose reaches peak values at 2 hours post-stress (Fanouraki et al., 2011). In large fish statistically significant lower values were observed in net than lift in S1 and S3, supporting the idea that net is a milder stressor than lift in those fish. In small fish, however, no differences were observed, suggesting that both methods are equally stressful.

Lactate is also commonly used as a stress indicator especially in cases of physical stress since it is the end-product of the anaerobic metabolism. In large fish there was a statistically significant effect of sampling, being lower in hooked animals than lifted in S1. No other differences were observed. In small fish a significant difference was observed only in S2, with lift resulting in lower levels than net. It seems therefore that there is no constant effect of sampling method in lactate values in both populations.

Haematocrit (HCT) showed no differences between sampling method apart from the lower levels in lift in both population in S3. These differences were possibly derived from seasonal and/or daily fluctuations in HCT rather than influenced by the sampling methods, since they were seen only in S3 in both populations.

Lysozyme activity was significantly reduced in fish sampled by the lift and net methods compared to hook in large fish. Past experiments have suggested that acute stress can quickly enhance the lysozyme activity of European sea bass while chronic stress may reduce it while complement was only affected after a longer lasting stress (data not published). Large fish showed lower antibacterial activity against both Gram-positive (lysozyme) and Gram-negative (complement) bacteria than smaller fish, significantly so concerning the lysozyme activity of fish lifted or netted and the complement of the netted fish.

In general, immunological data show high variability. Although, it was observed significant differences between the sampling methods for several immunological parameters (high NO in large fish lifted; high APA and lysozyme in large fish hooked) suggesting that some immune parameters are affected quickly by acute stress, it was difficult to recommend a specific way of sampling the fish in view to reduce the potentially adverse effects of the sampling technique on the immune status of the fish. However, lifting the net gave relatively stable results except for the nitric oxide concentration and may be considered as the best sampling method for immunological determinations in fish.

5. Conclusions

In conclusion, it seems that all methods tended to sample fish in a representative way in terms of morphometric characteristics. There was an indication that in the population of small fish, heavier fish were caught using the hook method, possibly due to social and hierarchical reasons. In terms of welfare, hook was the most rapid and less stressful method as suggested by the lower cortisol and glucose circulating levels. However, hook was not an effective sampling method since it was successful only in first trial. No consistent differences in physiological data were observed between net and lift in both populations. However, in large fish a tendency for more fish with low cortisol levels in net compared to lift methods suggests that net might be a milder stressor. Lactate and HCT showed no major influence by the sampling method.

References

Andrew, J.E., Noble, C., Kadri, S., Jewell, H., Huntingford, F.A. 2002. The effect of demand feeding on swimming speed and feeding responses in Atlantic salmon, *Salmo salar* L., gilthead sea bream *Sparus aurata* L., and European sea bass *Dicentrarchus labrax* L. in sea cages. Aquacult. Res. 33, 501-507.

Askey, P. J., Richards, S. A., Post, J. R., & Parkinson, E. A. (2006). Linking angling catch rates and fish learning under catch-and release regulations. *North American Journal of Fisheries Management*, 26, 1020–1029.

Dunier, M., Vergnet, C., Siwicki, A.K., Verlhac, V. 1995. Effect of Lindane Exposure on Rainbow Trout (*Oncorhynchus mykiss*) Immunity: IV. Prevention of Nonspecific and Specific Immunosuppression by Dietary Vitamin C (Ascorbate-2-polyphosphate). Ecotoxicol. Environ. Saf. 30, 259-268.

Ellis, T., Yildiz, H.Y., Lopez-Olmeda, J., Spedicato, M.T., Tort, L., Overli, O., Martins C.I.M. 2012. Cortisol and finfish welfare. Fish Physiol. Biochem. 38, 163-188.

Fanouraki, E., 2010. The study of stress and the evaluation of the non-invasive method of determination of free cortisol into the seawater in Mediterranean fish species, with emphasis in European sea bass, *Dicentrarchus labrax*. PhD Dissertation, University of Crete. pp. 212.

Fanouraki, E., Mylonas, C.C., Papandroulakis, N., Pavlidis, M., 2011. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. Gen. Comp. Endocrinol. 173(2), 313-322.

Fatira, E., Papandroulakis, N., Pavldis M., 2014. Diel changes in plasma cortisol and effects of size and stress duration on the cortisol response in European sea bass (*Dicentrarchus labrax*). Fish Physiol. Biochem. 40(3), 911-919.

Flik, G., Klaren, P.H.M., van den Burg, E.H., Metz, J.R., Huising M.O. 2006. CRF and stress in fish. Gen. Comp. Endocrinol. 146, 36-44.

Henry, M., Fountoulaki, E. 2014. Optimal dietary protein/lipid ratio for improved immune status of a newly cultivated Mediterranean fish species, the shi drum *Umbrina cirrosa*, L. Fish Shellfish Immunol. 37, 215-219.

Henry, M.A., Alexis, M.N., Fountoulaki, E., Nengas, I., Rigos, G. 2009. Effects of a natural parasitical infection (Lernanthropus kroyeri) on the immune system of European sea bass, *Dicentrarchus labrax* L. Parasite Immunol. 31, 729-740.

Henry, M.A., Nikoloudaki, C., Tsigenopoulos, C., Rigos, G. 2015. Strong effect of long-term Sparicotyle chrysophrii infection on the cellular and innate immune responses of Gilthead sea bream, *Sparus aurata*. Dev. Comp. Immunol., 51, 185-193.

Kokou, F., Rigos, G., Henry, M., Kentouri, M., Alexis, M. 2012. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. Aquaculture. 364, 74-81.

Louison, M. J., C. D. Suski**,** Stein, J.A. In Press. Largemouth bass use prior experience, but not information from experienced conspecifics, to avoid capture by anglers. Fisheries Management and Ecology. https://doi.org/10.1111/fme.12372.

Magnadóttir, B., Jónsdóttir, H., Helgason, S., Bjornsson, B., Jorgensen, T., Pilstrom, L. 1999. Humoral immune parameters in Atlantic cod (*Gadus morhua* L.): I. The effects of environmental temperature. Comp. Biochem. Physiol. B. 122, 173-180.

Pascoli, F., Lanzano, G.S., Negrato, E., Poltronieri, C., Trocino, A., Radaelli, G., Bertotto, D. 2011. Seasonal effects on hematological and innate immune parameters in sea bass *Dicentrarchus labrax*. Fish Shellfish Immun. 31, 1081-1087

Planas, J., Gutierrez, J., Fernandez, J., Carrillo, M., Canals, P. 1990. Annual and daily variations of plasma cortisol in sea bass, *Dicentrarchus labrax* L. Aquaculture 91, 171-178.

Rotllant, J., Ruane, N.M., Caballero, M.J., Montero, D., Tort, L. 2003. Response to confinement in sea bass (*Dicentrarchus labrax*) is characterized by an increased biosynthetic capacity of interrenal tissue with no effect on ACTH sensitivity. Comp. Biochem. Physiol. A. 136, 613-620.

Samaras, A., Papandroulakis, N., Costari, M., Pavlidis, M., 2016. Stress and metabolic indicators in a relatively high (European sea bass, *Dicentrarchus labrax*) and a low (meagre, *Argyrosomus regius*) cortisol responsive species, in different water temperatures. Aquacult. Res. 47(11), 3501-3515.

Glossary

AQUAEXCEL2020: AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020

.

Definitions

DO: Dissolved Oxygen

FCR: Feed Conversion Ratio

HCMR: Hellenic Centre for Marine Research

HSP: Heat Shock Proteins

HTC: Haematocrit

RMR: Resting Metabolic Rate

rpm: rounds per minute

SGR: Specific Growth Rate

Document information

EU Project N°	652831	**Acronym**	AQUAEXCEL[2020]
Full Title	AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020		
Project website	www.aquaexcel.eu		

Deliverable	**N°**	D6.6	**Title**	Sampling procedures in cages for physiological data
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Date of delivery	**Contractual**	11/2019 (Month 50)	**Actual**	11/2019 (Month 50)
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		CO Confidential, restricted under conditions set out in Model Grant Agreement		
		CI Classified, information as referred to in Commission Decision 2001/844/EC.		

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dd/mm/yyyy			Ex: first version/first review by WP leader etc/accepted version

Annex 1: Check list

Deliverable Check list (to be checked by the "Deliverable leader")

	Check list		**Comments**
BEFORE	I have checked the due date and have planned completion in due time		*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW		*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW		
	The contributors (authors) correspond to those indicated in the DOW		
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	I am using the AQUAEXCEL[2020] deliverable template (title page, styles etc)		*Available in "Useful Documents" on the collaborative workspace*
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AFTER	I have written a good summary at the beginning of the Deliverable		*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)		*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified		
	I have sent the final version to the WP Leader, to the 2nd Reviewer and to the Project coordinator (cc to the project manager) for approval		*Send the final draft to your WPLeader, the 2nd Reviewer and the coordinator with cc to the project manager on the 1st day of the due month and leave 2 weeks*

			for feedback. Inform the reviewers of the changes (if any) you have made to address their comments. Once validated by the 2 reviewers and the coordinator, send the final version to the Project Manager who will then submit it to the EC.