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Deliverable D5.2:

Calibration of physiological sensors technologies

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Executive summary

In order to establish a baseline of information regarding: i) muscular activity patterns linked to oxygen consumption, ii) mass specific standard metabolic rate (SMR), iii) maximum metabolic rate (MMR) of the target species gilthead sea bream, 125 fish were grouped in four size classes: group 1 included fish from 200 to 400 g; group 2 included fish from 400 to 600 g; group 3 included fish from 600 to 800 g and group 4 included fish from 800 to 1000 g.

The linear regression between weights indicated significant effect of fish mass on the U_{crit} data ($r = 0.529$, $p < 0.001$). In more detail, U_{crit} was greater in both group 1 and group 2 ($U_{crit} = 1.05$ and 1.02; $p < 0.05$, Tukey HSD) than in group 3 and 4.

The oxygen consumption rate of the four groups of fish was modelled using sigmoid curves in order to estimate the values of Standard Metabolic Rate (SMR), Maximum Metabolic Rate (MMR) and Aerobic Scope (AS). The SMR was relatively stable with the increase of fish size, while at the contrary MMR was considerably decreased with the increase of fish size, which resulted in the reduction of AS as a function of fish size from 510.7 to 307.6 of oxygen consumption (MO_2) from group 1 to group 4.

The activation pattern of the red muscle is described by a polynomial regression, while the white muscle shows very low activation until the swimming speed of 0.6 m/s. After this swimming speed, the contribution of the white muscles increases intensely, according to an exponential pattern, while the contribution of the red muscles is progressively reduced.

The acceleration of fish recorded with tailbeat accelerometer tag shows two different patterns as a function of swimming speed, depending of fish size. Although the two patterns are both exponential, from the step 0.6 to 0.9 m/s swimming speed, the acceleration of the tagged fish from group 1 displayed lower acceleration compared to other groups (Tukey HSD, $p < 0.005$). Since oxygen consumption and metabolic rates are different among the four groups, calibration of the accelerometer tags with oxygen consumption was performed for each group and a logarithmic model was used. The curve flattens with the increase in size due to the decrease of MMR.

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Introduction

Electronic sensors are significantly improving the possibility to monitor fish condition and are emerging as key sources of information for improving aquaculture management practices. The goal of this part of the project is to exploit the potential of Internet of Things (IoT) to address the challenges of a sustainable and resilient aquaculture system that ensures profitability, maintains healthy aquatic ecosystems and strengthens capacity for adaptation to climate change. To this purpose, we are going to set up a multiplatform tracking system for simultaneously monitoring the activity and physiology of fish, by using a wireless sensor system. Indeed, the fish farming industry needs instruments that can monitor in real time fish health and welfare objectively, without killing or disturbing the fish or interfering with the daily management.

Enhanced biological (e.g. behaviour, activity, energetic, feeding physiology) sensor data, collected by on-board electronic tags, will provide accurate fine-scale measurements of fish health and welfare during the large-scale demonstration activities in the project.

To this purpose, we firstly need to establish a baseline of information, for each of the target species, regarding: i) muscular activity patterns linked to oxygen consumption; ii) mass specific standard metabolic rate (SMR); iii) maximum metabolic rate (MMR). Then, the calibration of critical swimming speed (U_{crit}), electromyograms (EMG), oxygen consumption (MO_2) with accelerometer sensors gives the possibility to correlate each single swimming level to a metabolic state or to an activity index expressed as the EMG level. In particular, EMGs measure red (aerobic metabolism) and white (anaerobic metabolism) muscle activity. The EMG level at the U_{crit} speed represents the threshold limit of the aerobic muscular activity. In this way, the activity based energetic expenditure can be assessed and, consequently, the fish physiological status, as well as the relative cost of living for fish in their environment.

Specific technical information on the sensors for tracking the fish and the technologies used for measuring the swimming performances and energetic expenditure of fish are reported in Deliverable D5.1.

Hereinafter are reported the results of the laboratory tests carried out for the calibration of accelerometer sensors applied to the species gilthead sea bream (*Sparus aurata*).

Limitation due to covid-19

According to the DoA, we are committed to carry out the calibration of physiological sensors technologies mainly on the basis of available data, by reviewing the scientific literature.

When the FutureEUAqua project entered an operational phase we decided to enhance the informative power of the signals emitted by the sensors by performing *ex novo* the calibrations, for the species sea bream, sea bass and trout, in the COISPA laboratory, instead of relying on the scientific data available in the literature. This choice would have involved a considerable amount of extra work, therefore, deadline and program of D5.2 was rescheduled, in agreement with the project PO.

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Unfortunately, the spread of covid-19 has led to severe limitations, including the closure of the laboratories, since the beginning of the month of March 2020. For this reason, it was not possible to complete the calibration work for sea bass and trout.

Since, on the basis of currently available forecasts, a gradual reopening of the laboratories will begin in June 2020, by December 2020 we expect to complete and submit an updated version of the D5.2 with the results of the calibrations for sea bass and trout.

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Material and methods

All experiments were performed in accordance with Italian national legislation, EU recommendations (Directive 2010/63/EU) on fish welfare, and with the authorization of Health Ministry number 838/2019-PR.

Fish holding conditions

A total of 125 gilthead sea bream (*Sparus aurata*; mean weight ± SD = 616.12 ± 223,16 g) were used to model the oxygen consumption rates during swimming trials, muscle activity and the calibration of accelerometer tags with oxygen consumption (see Table 1).

Critical swimming tests and metabolic indexes estimation

Critical swimming tests measuring U_{crit} were performed using swimming chambers characterized by the capacity to generate laminar water flow with a constant water velocity profile. The trials were conducted by means of Loligo Systems 30 and 90 L swimming chambers according to manufacturer instruction regarding the fish size. The trials were controlled using the Loligo DAQ-M device, whereby a honeycomb screen was installed at the entrance of the swimming chamber to minimize turbulence and ensure that the water had a uniform velocity profile. The water flow speed was calibrated by means of a flow-meter, while the metabolic oxygen consumption rate was assessed using a polymer optical fiber (POF) oxygen probe inserted in the swimming chamber. The oxygen probe was connected to the Loligo WITROX oxygen instrument (fitted with a high-accuracy temperature sensor) to sample oxygen variations during closed respirometer trials. Finally, the swimming chambers were housed in a buffer tank to guarantee precise temperature control.

The swimming tests were conducted by imposing a swimming speed ramp (0.1 m s^{-1}) at constant time intervals (10 min) until fatigue was reached (Carbonara et al. 2010). U_{crit} values were estimated according to Brett et al. 1964, and corrected for solid blocking effects (Smit et al. 1971; Kline et al. 2015). Fish were grouped in four size classes (Table 1): group 1 included fish from 200 to 400 g; group 2 included fish from 400 to 600 g; group 3 included fish from 600 to 800 g; and group 4 included fish from 800 to 1000 g. Among the 125 fish, 45 were challenged in U_{crit} test, without using any device (i.e. sensors), to trace a baseline of oxygen consumption and estimation of metabolic rate (control), 36 were challenged using hard-wire electromyograms (EMG) for modelling the muscle activity (EMG) and 44 were challenged using accelerometer tag (TAG) for the calibration of accelerometer tag with the oxygen consumption (Table 1).

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Table 1. Morphometric measures (mass and total length (mm)) of specimens involved respectively in the oxygen consumption rate modelling (control), the assessment of muscle activation pattern (EMG) and, finally, the group of fish used for the tag calibration trials (TAG).

Group	Type	n	Fish body mass (g)	Total length (mm)
200-400 g	Control	6	348 ± 27.63	232.62 ± 10.46
	EMG	9	325.17 ± 45.86	277 ± 10.59
	TAG	13	301.23 ± 65.15	268.74 ± 16.06
	Total group 1	**28**	**318.95 ± 54.80**	**263.65 ± 47.99**
400-600 g	Control	15	474.01 ± 59.07	303.07 ± 10.26
	EMG	9	477.99 ± 44.58	307.02 ± 10.67
	TAG	5	520.8 ± 67.65	316.4 ± 23.04
	Total group 2	**29**	**483.31 ± 57.21**	**306.59 ± 13.6**
600-800 g	Control	12	705.96 ± 69.76	360.92 ± 14.44
	EMG	8	679.61 ± 53.33	361.875 ± 16.42
	TAG	13	701.6 ± 58.07	359.69 ± 9.26
	Total group 3	**33**	**697.86 ± 60.6**	**360.67 ± 12.79**
800-1000 g	Control	12	895.9 ± 58.48	386.08 ± 11.45
	EMG	10	882.77 ± 64.31	388.9 ± 8.48
	TAG	13	881.59 ± 57.08	389.15 ± 7.93
	Total group 4	**35**	**886.83 ± 58.27**	**388.03 ± 9.25**

The models of the oxygen consumption rate as a function of water speed were used to estimate the values of the metabolic indices: standard metabolic rate (SMR), maximum metabolic rate (MMR), and the aerobic scope (AS). SMR indicates the minimal energetic demand required by the fish for basal metabolic activities in resting conditions and was extrapolated by swimming models at speed = 0 (Chabot et al. 2016). MMR indicates the maximum oxygen consumption rate sustained during exhaustive swimming exercises (U_{crit}) (Norin and Clark 2016). The numerical difference between MMR and SMR indicates the energetic expenditure available to support both locomotive and physiological activities: that is, AS (Fry 1947; Brett 1972).

Hard-wire electromyogram (EMG): muscle activity

A sub-group of 36 fish (Table 1) was randomly selected to assess the activation patterns of both red and white muscles by means of EMG during swimming trials. The fish were fasted 24 h before starting the trial to ensure a post-absorptive state (McFarlane et al. 2004) and were anesthetized using 30 mg L^{-1} hydroalcoholic solution of clove oil (eugenol) (Massee et al. 1995; Zupa et al. 2015). During the surgery, the gills were continuously irrigated with anaesthetic solution. Two pairs of plastic-coated stainless-steel wire electrodes (0.1 mm thin and 1 mm long) were surgically implanted subcutaneously using syringe needles, in both lateral red musculature and white muscle (in the same location but approximately 1 cm below the surface). The two electrodes of each pair were placed at least 10 mm apart from one another to avoid potential contact during contraction (Cooke et al. 2004). The wires

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were sutured to the left side of the body to minimize entanglement. The EMG signals were sampled and digitally converted using the National Instruments USB-6009 device (sample rate: 48kS/s), amplified with Grass P511 preamplifiers, filtered, and root-mean-square (RMS) averaged using LabVIEW SignalExpress.

Calibration of tailbeat accelerometer tags (TAG)

A sub group of 44 specimens (Table 1) was randomly selected to calibrate the swimming activity, measured by tailbeat accelerometer tags and oxygen consumption during U_{crit} tests. The calibration was conducted using V9AP acoustic accelerometer tags manufactured by VEMCO and programmed to measure, with a sampling rate of 10 Hertz, the acceleration over two axes (X and Z) (Brownscombe et al. 2018), excluding the Y-axes of the backward/forward movements. Accelerometers transmit the tag ID and the coded values corresponding to the acceleration with an average delay of 30 sec. The acceleration data were stored in the memories of submergible acoustic receivers (Vemco VR2W). The fish were fasted 24 h before the surgery (McFarlane et al. 2004) and were then anesthetized using 30 mg L^{-1} of hydroalcoholic clove oil solution, as for EMG procedure. Afterwards, the tag was implanted into the body cavity through a 1.5 cm incision. A calibration model of the oxygen consumption rate as a function of the tailbeat tag activity was fitted, with the best model selected (see details in the Statistical Analyses section).

Statistical Analyses

Statistical analyses were performed using R 3.1.0 software (R Development Core Team 2018). All statistical analyses were carried out at the 95% level of significance.

For the critical swimming tests, linear regression was applied between U_{crit} data and fish weight. Moreover, ANOVA test was performed, followed by the HSD Tukey post-hoc test, to compare the U_{crit} a function of the four different size groups. Subsequently, the energetic expenditure of swimming observed in the sea bream was assessed for each size group, estimating four different models of the oxygen consumption rate, as a function of the swimming speed, all using a sigmoid model. The sigmoid model was based on the following formula:

$$Y = \frac{A_{sym}}{1 + e^{\frac{x_{mid} - U_m}{scal}}}$$

where *Asym* is a numeric parameter representing the asymptote; *xmid* is a parameter representing the X value at the inflection point of the curve, when Y=Asym/2; *scal*: is a scale parameter for the X-axis (swimming speed, U_m); and U_m represents the speed of the water in the tube without the fish.

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Both red and white muscle activation patterns were modelled using nonlinear models. In particular, the red muscle EMG data were modelled using a polynomial model, while the white muscle was modelled using an exponential model.

Finally, the calibration model of the oxygen consumption rate was fitted as a function of the tailbeat tag activity (i.e. acceleration), selecting the optimum model from the linear, exponential, and logarithmic models and choosing that with the lowest AIC value for each group (Akaike 1973). For each group, the relationship was modelled using logarithmic model.

Results

Critical swimming test and metabolic indexes estimation

The linear regression between weight indicated significant effect of fish weight on the U_{crit} data ($r = 0.529$, $p < 0.001$). In more details, U_{crit} was greater in both group 1 and group 2 ($U_{crit} = 1.05$ and 1.02; $p < 0.05$, Tukey HSD) than in group 3 and 4 ($U_{crit} = 0.95$ and 0.90; Figure 1).

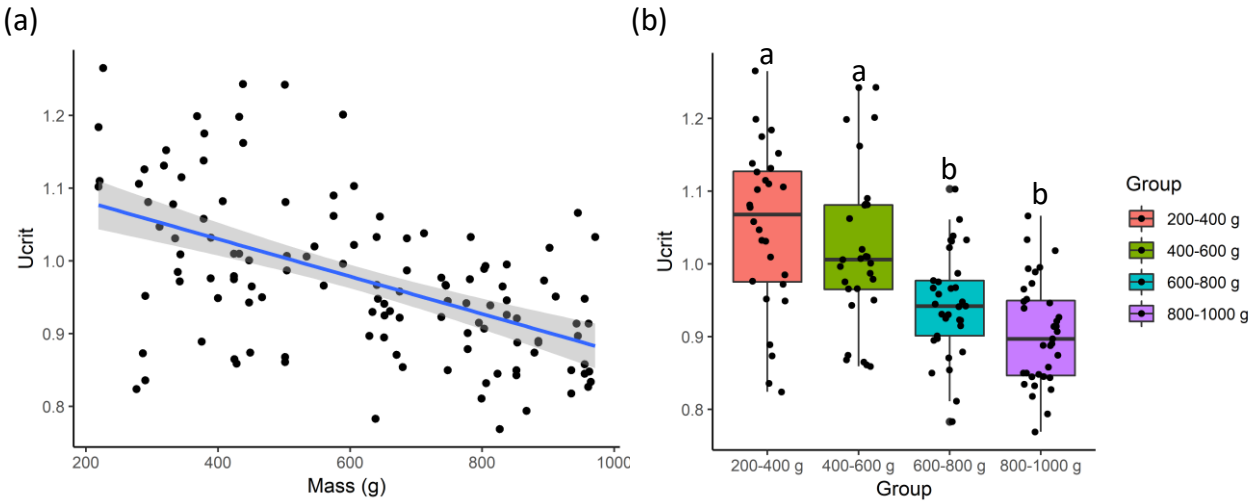

Figure 1. U_{crit} (m/s) values estimated during swimming trials. (A) Relationship between weight (g) and U_{crit} ($p < 0.001$); (B) U_{crit} (m/s) depending on the four size classes of fish. The central line of the boxplot indicates the median and the boxes the quartiles, with the whiskers covering 95% of the values. All values for each group are represented by points. Different letters indicate significant differences between groups (Tukey HSD post-hoc test, $p < 0.05$).

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The oxygen consumption rate of the four groups of fish was modelled using sigmoid curves (see Figure 2). The respective models' parameter estimations, standard errors, and associated p-values are reported in Table 2.

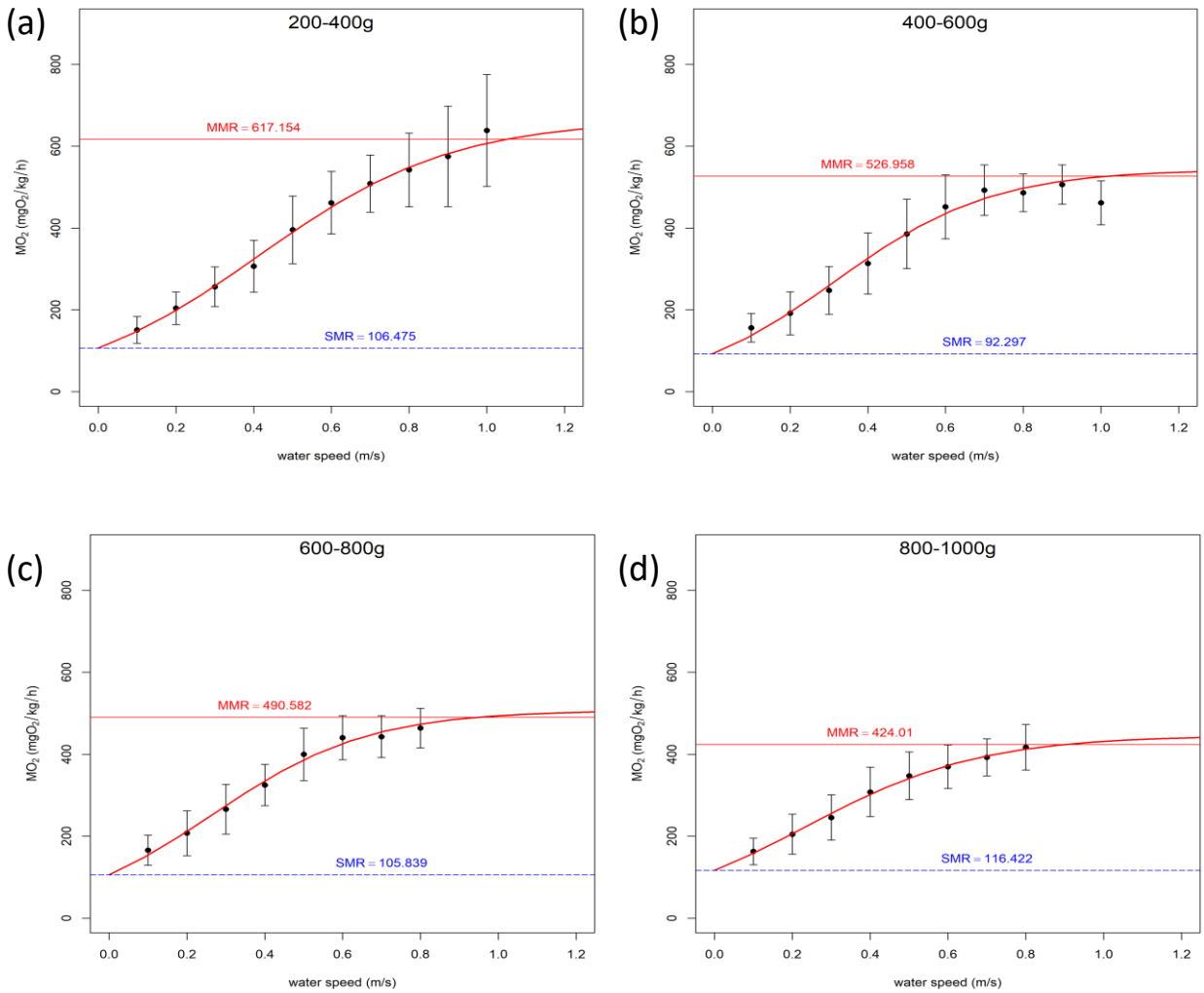

Figure 2. Oxygen consumption rate (MO_2 (mgO_2/kg/h)) depending on water speed (m/s) for (a) group 1, (b) group2, (c) group 3 and (d) group 4. The standard metabolic rate (SMR, blue) and maximum metabolic rate (MMR, red) are reported for each group. Estimates and standard errors of the different models are available in Table 2.

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Table 2. Summary of the estimates, standard error and the associated p-value for parameters of the sigmoid models performed for each group. Associated R^2 of the model are reported for each group.

	Group 1 (R^2 = 0.80)			**Group 2** (R^2 = 0.79)		
Parameters	Estimate	St. Error	p-value	Estimate	St. Error	p-value
Asym	665.10	37.61	< 0.001	542.7	20.21	< 0.001
Xmid	0.41	0.03	< 0.001	0.32	0.02	< 0.001
Scal	0.25	0.02	< 0.001	0.20	0.02	< 0.001
	Group 3 (R^2 = 0.78)			**Group 4** (R^2 = 0.71)		
Parameters	Estimate	St. Error	p-value	Estimate	St. Error	p-value
Asym	506.6	20.5	< 0.001	445,9	24.7	< 0.001
Xmid	0.27	0.02	< 0.001	0.23	0.03	< 0.001
Scal	0.2	0.02	< 0.001	0.22	0.03	< 0.001

The estimated values of Standard Metabolic Rate (SMR), Maximum Metabolic Rate (MMR) and Aerobic Scope (AS) are reported in Figure 3. The SMR is relatively stable with the increase of fish size, while at the contrary MMR is considerably reduced, which results in the reduction of AS as a function of fish size from 510.7 to 307.6 MO_2 from group 1 to group 4 (Figure 3).

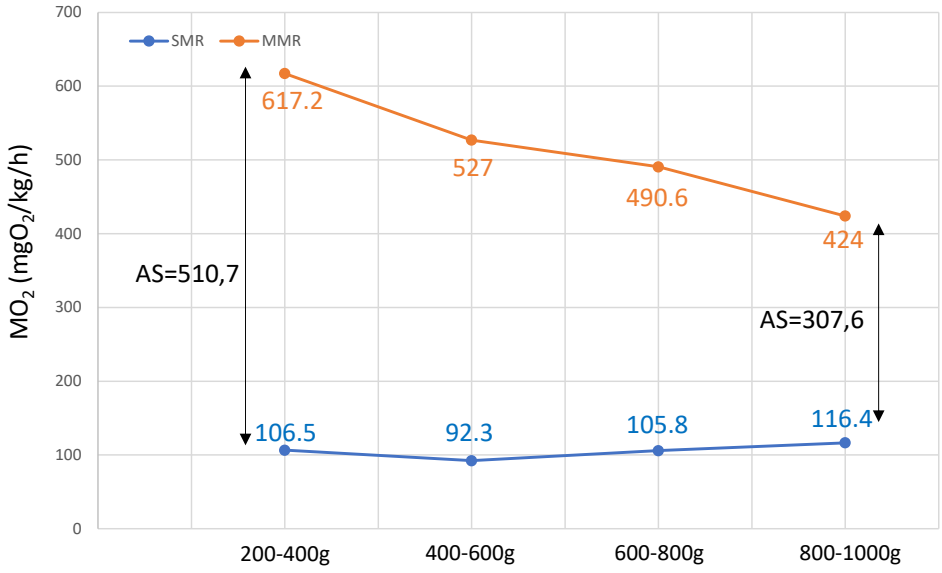

Figure 3. Estimates of the standard metabolic rate (SMR), maximum metabolic rate (MMR) and aerobic scope (AS), (MO_2 (mgO_2/kg/h)) as the function of size groups based on sigmoid models.

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Muscle activity

The pattern of the recruitment of both red and white muscle fibres is somewhat species-specific. In our test, the activation pattern of the red muscle is described by a polynomial regression, while the white muscle shows very low activation until a speed of 0.6 m/s (Figure 4). After this swimming speed, the contribution of the white muscle increases strongly according to an exponential pattern, while the contribution of the red muscles is progressively reduced (Figure 4). This result highlights that for the estimation of the overall aerobic scope, not only the energy of the red muscle should be taken into account. Indeed, fine-tuning the contribution from white muscle would permit a more accurate estimate of the energetic budget available for the fish to be used in compensatory activity in response to stressors, such as husbandry protocols.

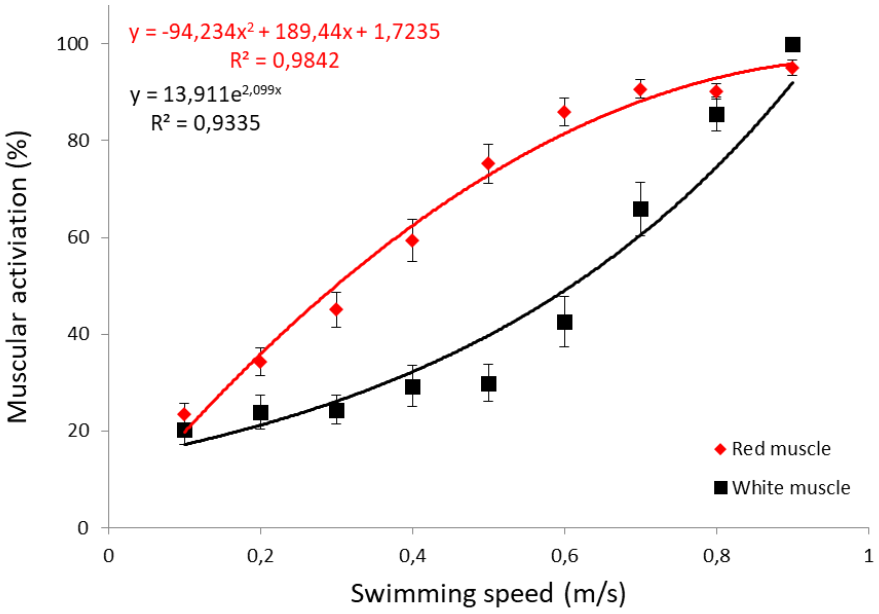

Figure 4. Muscular activation (%, mean ± s.e.m.) of the red (red points) and white muscles (black points) as function of the swimming speed (m/s). Red muscle activation was fitted using polynomial regression while white muscle was fitted using exponential model ($p < 0.001$ for both). Equations of model and associated R^2 were reported for red muscle (red colour) and for white muscle (black colour).

Calibration of tailbeat accelerometer tags

The acceleration of fish recorded with a tailbeat accelerometer tag shows two different patterns as a function of swimming speed, depending of fish size (Figure 5). Although the two patterns are both exponential, from the step 0.6 to 0.9 m/s swimming speed, the acceleration of the tagged fish from group 1 displayed lower acceleration compared to the other groups (Tukey HSD, $p < 0.005$) (Figure 5).

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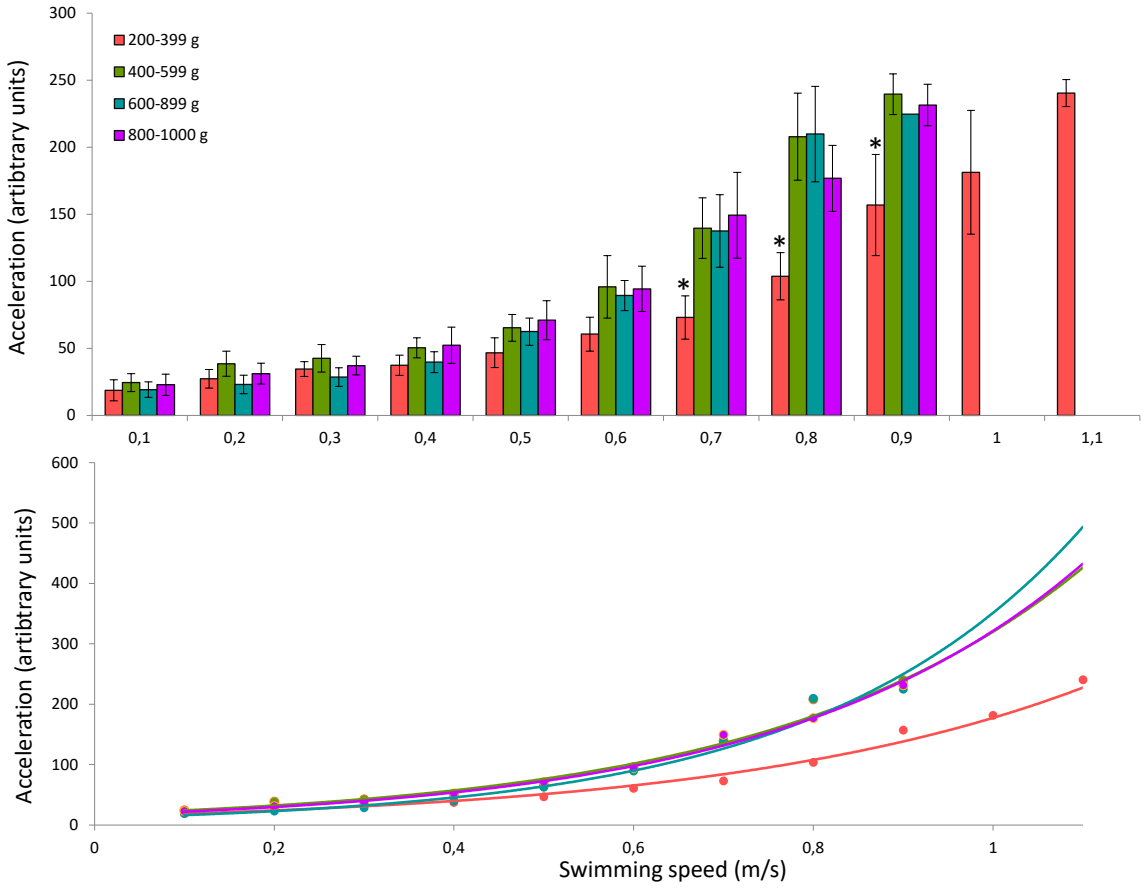

Figure 5. Acceleration (arbitrary unit) recorded with accelerometer tag as function of the swimming speed (m/s) for each group. * indicates significant difference from group 1 (200-400 g) with other groups from the same swimming speed step (Tukey HSD, $p < 0.005$).

Since oxygen consumption and metabolic rates are different among the four size groups (see Figures 2-3), calibration of the accelerometer tags with oxygen consumption was performed for each of the groups, and a logarithmic model was used (Figure 6). The curve flattens with the increase in size due to the decrease in MMR reported above. The model equations are shown in Figure 6, while parameter estimates and standard errors are shown in Table 3.

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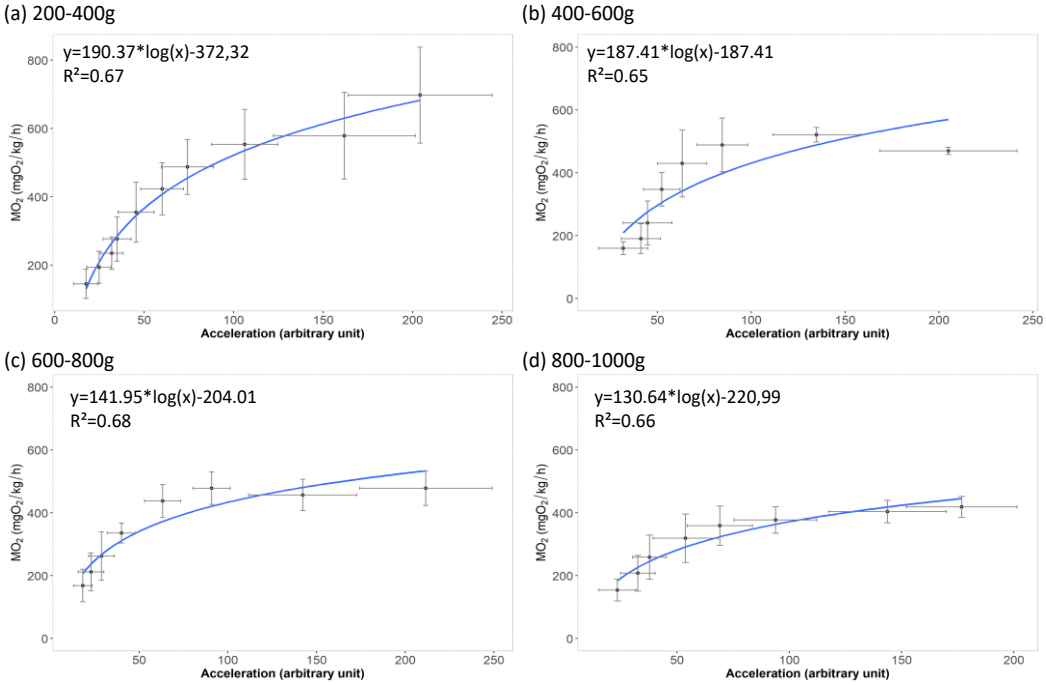

Figure 6. Calibration of acceleration obtained from tag and oxygen consumption rate for the (a) 200-400 g, (b) 400-600 g, (c) 600-800 g and (d) 800-1000 g. The blue line represents the tailbeat tag calibration using logarithm model. Points represent the observed tail acceleration (arbitrary unit, mean ± sd) and the relative MO_2 values ($mgO_2/kg/h$, mean ± sd) at each speed interval of the U_{crit} test. Equations of the model and the associated R^2 are reported for each group.

Table 3. Summary of the estimate, standard error and the associated p-value for parameters of the logarithm models used for the calibration acceleration with oxygen consumption for each group. Associated R^2 of the model are reported for each group.

	Group 1 (R^2 = 0.67)			**Group 2** (R^2 = 0.65)		
Parameters	Estimate	St. Error	p-value	Estimate	St. Error	p-value
(Intercept)	-372.32	48.33	< 0.001	-426.76	93.66	< 0.001
Log(acceleration)	190.37	12.16	< 0.001	187.41	22.26	< 0.001
Parameters	**Group 3** (R^2 = 0.68)			**Group 4** (R^2 = 0.66)		
(Intercept)	-204.01	44.63	< 0.001	-220.99	45.47	< 0.001
Log(acceleration)	141.95	11.21	< 0.001	130.64	11.37	< 0.001

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Discussion and conclusion

The results of the tests carried out with sea bream demonstrate that the fish size represents a critical factor for both the swimming activity and its relative energetic costs. Indeed, smaller fish showed a more efficient oxygen consumption rate than larger ones at any given swimming speed. Also the critical swimming speed (U_{crit}), which is an indicator of the mass-specific costs of locomotion, resulted positively correlated with the fish size.

Rearing procedures at aquaculture facilities (e.g. stocking density, quality of feed, etc.) may be sources of stress, and are thus responsible for an increased energy demand. Electromyograms record bio-electrical voltage changes and duration of muscle tension, that are both indicators of the energetic demand of the fish. The value of EMG at maximum critical swimming, derived from the U_{crit} calibration, represents a threshold limit of red muscle energy expenditure, that is the maximum sustainable aerobic activity for a fish (Carbonara et al. 2014). The higher red muscle activity, close to the U_{crit}, is linked also to a higher use of the anaerobic substrates, which represent the reserve energies (Lembo et al. 2007). Less availability of anaerobic energetic reserves has consequences for the reactivity of stress systems, reflecting on a reduced ability of the fish to compensate stressful events. For this reason, data from electromyograms have been also used as an indicator of the fish well-being (Lembo, et al. 2008).

Fish were surgically implanted with tailbeat accelerometer tags to measure the acceleration of the fish during the U_{crit} test. The animal's acceleration signal is measured in terms of m/s^2 and is a vector quantity that is a result of measuring acceleration on two axes (X,Z) by a tailbeat algorithm. Our objective was to find a calibration model of the tailbeat tag activity as a function of the i) critical swimming speed, ii) oxygen consumption and metabolic rate, iii) electromyograms.

In this way, we will be able to provide accurate fine-scale measurements of the fish physiological state (e.g. stress conditions), during the large-scale demonstration activities, by using the sensor data collected and transmitted, in real time, by the electronic tags implanted to the fish.

The above considerations are species specific for sea bream. As soon as the ongoing tests on sea bass and rainbow trout will be concluded, a comparative assessment of the ability to compensate stress events, among the three species, will be carried out.

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