**Prolonged emersion of *Solea senegalensis*, Kaup 1858, for its
application in transport**

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Running title: Waterless transport of Senegalese sole.

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Abstract

This study aimed to determine the maximum time of emersion of Senegalese sole (*Solea senegalensis*) specimens to form the basis for a dry sole transport procedure. For this purpose, 56 specimens (922.33 ± 29.52 g body weight) were individually placed in polystyrene boxes on a wet cloth, maintaining constant temperature (15 °C) during emersion trial. Specimens ($n = 7$ per group) were sampled for plasma and mortality was monitored at different points (0, 2, 6, 12, 20, 28, 36, and 44 hours of emersion), and several stress (cortisol, glucose and lactate) and metabolic (triglycerides, proteins, amino acids) parameters were assessed. After sampling, each experimental group was placed in recovery tanks for the assessment of delayed mortality and feeding performance after emersion trial. Transport periods longer than 28 hours produced high stress level indicated by i) significant increase in circulating cortisol and lactate, and ii) glucose reserves mobilization. These alterations originated physiological problems during the emersion trial that induced mortality as well as problems for recovering correct feeding performance of specimens. These results suggested that a waterless transport is possible without direct or delayed mortality up to 28 hours.

Introduction

Live aquatic animals transport and distribution is becoming an important part of the aquaculture industry, and all the aspects focused on cost reduction, product quality improvement, or reducing health problems, contribute significantly to a better development of this sector (Berka, 1986). The most common is the transport of larvae or fingerlings between different production units, but occasionally it is necessary to move larger specimens or breeders. Transport of large live individuals is important in the management of broodstocks in the companies or research centers, and in the

51 maintenance of the product quality and hence the price (Southgate, 2008). In order to
52 maintain the organoleptic qualities, product degradation must be avoided. Degradation
53 can be produced by microorganisms but also by autolysis. In the last case, product
54 spoilage occurs due to endogenous enzymes, and a decrease in the final product quality
55 is provoked (FAO, 1998). By transporting live animals, autolysis is prevented and
56 quality is preserved. This improvement in quality could be correlated with higher price
57 in the market; Japan is one of the most important examples where the price of live fish
58 is higher than that of fresh fish (Takashima, 1996).

59 Among all the fish transport systems, the most common way is in water, either through
60 an open or closed system (Berka, 1986), but the transport of specimens without water
61 has been successfully used in some species such as turbot (*Scophthalmus maximus*)
62 (Caillens, 1996; Liu, Xue, Yin & Liu 2009). Even so, the water transport presents some
63 drawbacks, such as a high technical requirements, higher cost, or problems with water
64 and equipment used for transport related to pathologies dissemination (Southgate,
65 2008). By contrast, transport without water is easy to perform, inexpensive, do not
66 generate waste problems and allow bigger load amount (Yin Liu, Liang & Jiang 1995),
67 although it may not be used for all species. Most of the shellfish are transported without
68 water, and sea products such as lobster, shrimp, oyster or scallop are maintained at low
69 temperatures during transportation without need for water. In fish, some trials have been
70 performed successfully mainly in turbot (Caillens, 1996; Liu et al., 2009), and there
71 have been also positive results in non-flat species as the Giant grouper, *Epinephelus*
72 *tauvina* (Comandante, 2005). However, the different ways of addressing waterless
73 transport, make it necessary to develop the technique for each species.

74 Another important aspect in relation to live animals transport is that related to animal
75 welfare. European Union regulates the terms in which the transport of live animals

should be performed (Directive (EC) 1/2005, of December 22, 2004 on the protection of animals during transport and related operations). Thus, this text aims to prevent injury or undue suffering to animals and to ensure that they have appropriate conditions that meet their needs. One way to determine the welfare status of specimens during transport is to assess stress level to which animals are subjected. The most accepted method to determine stress levels is the study of plasma cortisol as well as other plasmatic parameters (glucose and lactate) (Wendelaar Bonga, 1997; Barton 2002).

The aim of this study was to define a maximum emersion time in specimens of *S. senegalensis* of commercial size or breeders, for its application in transport, determine stress levels produced by this activity, as well as the short-term effects of prolonged emersion (possible delayed mortality, first food intake, and feeding performance after stress).

Material and Methods

Experimental procedures

The experiment was carried out in the Marine Aquaculture Plant “El Bocal” of the Spanish Institute of Oceanography (IEO), in Santander (Spain). A total of 56 individuals of *S. senegalensis* (922.33 ± 29.52 g body weight, and 39.80 ± 0.47 cm body length) obtained from a same age and origin stock, were used in the experiment. Initially, all the group (n=56) was on a 3 m³ tank located inside an industrial warehouse, in open flow circuit, with a water renovation rate of 1.2 m³/hour and constant moderate aeration. An artificial photoperiod of 16 hours light, and 8 hours dark was used throughout the entire year. The light intensity was reduced using mesh shading over the tanks that allowed a maximum light intensity at the water surface of 50 lux. Fish were

fasted 72 hours before the emersion tests. The specimens were randomly divided into 8 groups of 7 individuals based on the statistical study of minimal sample number to achieve the study objectives. During the emersion period, the specimens were placed in 2 cm thick polystyrene boxes (59x39x13 cm) on moistened cloths to maximize humidity levels (1 fish per box). The non use of anesthetics during emersion to reduce stress or distress attended to approach baseline parameters without interference of any substance. All the boxes were placed in a room with constant temperature (15 °C) during emersion time. At the beginning of the experiment, 7 individuals were blood sampled to establish the control levels of different plasmatic parameters assessed in this study (t=0 hours). Then, on 2, 6, 12, 20, 28, 36 and 44 hours of emersion, groups of 7 individuals were also blood sampled. No longer emersion periods were tested to avoid mortality since analogous species (*S. maximus*) can survive up to 44-60 hours in similar conditions (Caillens, 1996; Liu et al., 2009). After sampling, each experimental group was introduced into an aerated recovery tank (volume 1.125 m³) under the culture conditions described above to evaluate their performance after the emersion period and the possible delay in mortality. For this purpose, survival rate and food intake were recorded during four weeks after the emersion trial. For the valuation of food intake after the emersion test, fish were daily feed late in the afternoon with one pellet per fish (Vitalis Repro 9 Skretting S.L.), and the detection of uneaten pellets remaining in the tank (Rubio, Sanchez-Vazquez & Madrid 2003) was conducted the next day early in the morning to avoid the pellets got rid in the water. Tanks were fed from Monday to Thursday according to the facilities feeding routines. The percentage of food intake was calculated as the number of pellets each group ingested by the total of pellets offered.

Blood sampling procedures

For blood sampling, each individual alive was removed from the box, and the blood was withdrawn from the caudal vein using 1 mL heparinized syringes. Sampling began at the times indicated above, but no fish was removed from the box until having finished the previous sample. Moreover, once opened the box each blood sample was taken in less than three minutes to avoid plasma cortisol and glucose enhancement due to handling (Arjona, Vargas-Chacoff, Ruiz-Jarabo, Martín del Río & Mancera 2007). The extracted blood was transferred to 1,5 mL eppendorfs tubes containing 10 µL of heparin and centrifuged (3000 x g for 15 min. at 4 °C) to obtain plasma, that was stored at -80 °C until further analysis.

The experiment was performed following the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals. The study was conducted under the activities of the project AGL2013-48835-C2-1-R (Ministerio de Economía y Competitividad, Spain) and approved by the Ethical Committee Competent Authority (Junta de Andalucía Autonomous Government) under the reference 28-04-15-241.

Blood analytical procedures

Glucose, lactate and triglycerides concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides ref. 1001311) adapted to 96-well microplates. Plasma protein concentrations were measured on a 50-fold plasma dilution using the bicinchoninic acid method with the BCA protein kit (Pierce P.O., Rockford, USA), with bovine serum albumin serving as standard. Free amino acid levels were assessed colorimetrically on a 75-fold plasma dilution using the ninyhydrin method (Rosen. 1957). All the assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc.,

Winooski, USA) controlled by KCjunior™ software. Standards and samples were measured in duplicate.

Plasma cortisol levels were measured by Enzyme Immune-Assay (EIA) using microtiter plates (MaxiSorp™, Nunc, Roskilde, Denmark) as previously described by Martos-Sitcha *et al.* (2014) for other teleost species. Steroids were extracted from 5 µL of plasma in 100 µL RB (10 % v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01 % w/v NaN₃, 2.34 % w/v NaCl, 0.037 % w/v EDTA, 0.1 % w/v BSA (Bovine Serum Albumin)) and 1.2 mL methanol (Panreac), and evaporated during 48-72 hours at 37 °C. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate.

Statistics

Results are presented as means ± SEM. After normality and homogeneity of variance were checked (Kolmogorov–Smirnov and Levene tests, respectively), comparison between groups was analysed as appropriate using one-way analysis of variance (ANOVA) taking the time of emersion as main factor, followed by post-hoc comparison with Tukey's test. Significance was taken at $P < 0.05$.

Results

No mortality was observed during the different times of emersion excepting at 44 hours, where 66.7 % of the specimens died (Table 1). Delayed mortality, registered during the

24 hours after introduction of specimens in recovery tanks, was 42.8 % and 16.7 % in the group of 36 and 44 hours of emersion respectively (Table 1). In addition, the group emerged during 20 hours registered 1 individual death (14.3 % mortality) fifteen days after the emersion trial (Table 1).

The groups with significantly higher levels of ingestion ($p < 0.05$) were those maintained for 2, 6, 12, 20 and 28 hours of emersion (Table 2), ranging between 26.3 and 53.3 % of intake. In addition, the group with a significantly lower level of intake was 44 hours ($p < 0.05$) in which food ingestion was not observed during 4 weeks (Table 2). Groups of 0 and 36 hours showed intermediate levels of intake (Table 2). No food intake was observed in any group until seven days after the emersion trials (Table 2).

Plasma cortisol levels showed a progressive increase throughout the emersion time, although no significant difference respect to control was observed prior to 12 hours (Figure 1A). In addition, the highest levels of this hormone were found in those specimens maintained during 28, 36 and 44 hours out of water, with more than 7-fold increase when compared with control group (0 hours). Moreover, glucose levels enhanced significantly in the groups of 2, 6 and 12 hours at emersion, although no differences in the remaining groups were observed (Figure 1B). Furthermore, lactate showed its highest values in those groups maintained during 12 and 20 hours at emersion ($p < 0.05$), decreasing its values after this time (Figure 1C).

In addition, Figure 2 shows plasma values of triglycerides, total proteins and free amino acids after different hours of emersion. Thus, triglycerides levels enhanced significantly in those groups maintained during 2 and 28 hours at emersion (Figure 2A), whereas proteins significantly increased its levels in the groups of 20 and 44 hours (Figure 2B). Finally free amino acids levels decreased its values after 6 hours of emersion and recovered basal levels at 44 hours (Figure 2C).

199 Discussion

200 This study was aimed to determine the optimum emersion time for *S. senegalensis*
201 specimens (about 1000 g body weight) kept in polystyrene boxes on a wet cloth at 15
202 °C. For this purpose, mortality rates during and after emersion trial as well as feeding
203 performance during a period of four weeks after trial were assessed. In addition,
204 evaluation of stress system activation was carried out by analysis of different plasma
205 stress (cortisol, glucose, and lactate) and metabolic (triglycerides, proteins and free
206 amino acids) parameters in order to establish the effect of transport in terms of animal
207 welfare. The results will be discussed in relation to its application in transport processes
208 of *S. senegalensis* specimens of large size or/and breeders.

209 In the case of transport in water, commonly oxygen-enriched, a high percentage of
210 mortality could occur if monitoring systems or oxygen supply fail and the dissolved
211 oxygen is consumed completely (Berka, 1986). The principal advantage of waterless
212 transport, is that oxygen level available for fish does not change along the time, and
213 only depends on gill epithelium integrity, since the oxygen content in air is much higher
214 than in saturated water (Carter, 1975). Oxygen exchange and uptake out of water is
215 possible, while secondary folds on the filaments of the gills do not collapse against each
216 other (Carter, 1957). Even so, the putative mortality produced in cases of waterless
217 transport seems to be mainly related to metabolites excretion inhibition, resulting in
218 excessively high plasmatic content of ammonia nitrogen (Liu et al., 2009).

219 Survival results obtained in this study, allowed establishing a safe period of 28 hours for
220 waterless transport in *S. senegalensis* specimens without direct or delayed effect on
221 mortality under those conditions used in our trial. Even so, the mortality observed in the
222 group of 20 hours (1 specimen) could not be directly associated to our experimental

design, because it was a single death recorded 15 days after the emersion trial. Similar survival results have been obtained in other studies performed in *S. maximus* (Calliens, 1996; Liu et al., 2009) or *Dormitator latifrons* (Chang, 1984). Results obtained by Chang (1984) also show the importance of using methodologies that ensure a high level of humidity during air exposure, and how this positively affects the emersion times allowed without mortality. In addition, studies with *S. maximus* indicated that longer times of emersion were achieved without mortality when low temperatures were used, suggesting that environmental temperature could be important factor to be considered in waterless transport. The metabolism decrease induced by low environmental temperature, and the consequent attenuation of oxygen consumption and/or waste metabolite production could explain the longer time of emersion without mortality (Clarke and Johnston, 1999). However, other species such as Pacific halibut, *Hippoglossus stenolepis*, do not exhibit the same air exposure tolerance, and significant mortality occurred after 40 min and 60 min of air exposure in 1 and 2 years specimens, respectively (Davis and Schreck, 2005).

In terms of behavior, no specific observations were conducted, however at the time of sampling (20 minutes per sampling point) specimens showed no continuous fluttering or stirring during the experiment, and only some low-intensity isolated flutter happened. Therefore, at these time points (approx 10% of the duration of the experiment) no notable behavior changes related to emersion were observed. However, chronic or unavoidable stressors may deny this function and so behavioral changes may become maladaptive (Ashley, 2007).

Feeding patterns described under our experimental conditions reflected substantial variability, since the minimum level of management (group 0 hours) inhibited food intake during one week at least. In general, stress response results in an immediate feed

intake decrease (Wenderlaar Bonga, 1997). However, Bernier *et al.* (2004) observed how food intake gradually increased in goldfish (*Carassius auratus*) using a low cortisol enriched diet and how this increase was significantly elevated between days 9 and 21, comparing with a non-supplemented with cortisol diet. In addition to that, Salas-Leiton *et al.* (2008 and 2010) showed not only an increase in food intake in *S. senegalensis* juveniles but also higher cortisol levels associated with high stocking densities (30 Kg m⁻²). Thus, the results obtained in the present work confirm both patterns, related to i) an initial inhibition in food intake, and ii) a subsequent increase of the global intake significantly higher in those groups maintained out of water (stressed), which did not register mortalities (G.2, 3, 4, 5 and 6).

Regarding with stress parameters, although these values obtained are consistent with the trends of the parameters along the time, the levels obtained in the 44 hours group must be considered with caution when compared with the other experimental groups, since only 2 individuals were alive at the time of blood sampling, and one of them died within 24 hours after sampling. It has been demonstrated that plasmatic biochemical parameters are altered in fish under comatose condition (Lian-Tien, Guan-Ru & Ching-Fong 1994).

Cortisol is the most widely stress indicator used in fish (Wenderlaar Bonga, 1997). Cortisol enhancement was observed after 12 hours emersion, while group submitted to 2 hours of emersion showed similar values that control group. Hormonal levels observed in the group emerged during 6 hours are comparable to those obtained by Oliveira *et al.* (2013) using *S. senegalensis* specimens (413± 36 g body weight) in basal conditions (unstressed), which ranged from 5 to 35 ng/mL over a 24 hour cycle, indicating the existence of a circadian rhythm for plasma cortisol values in this species. In addition, and due to the existence of a daily rhythms for the different endocrine factors related to

hypothalamus-pituitary-interrenal axis as well as in the acute stress responses in *Solea senegalensis* (López-Olmeda, Blanco-Vives, Pujante, Wunderink, Mancera & Sánchez-Vázquez 2013), the influence of time of the day for starting emersion trials will be necessary to be considered in future studies. According to these results, we can assume that transport without water could be performed without inducing stress up to 12 hours, first time when cortisol level enhanced significantly respect to control.

In juveniles of *S. senegalensis*, acute stress induced a first stress peak between 5 minutes and 2 hours after the stress challenge (Wunderink, Engels, Halm, Yufera, Martinez, Rodriguez-Flik, Klaren & Mancera 2011; Costas, Conceicao, Aragao, Martos, Ruiz-Jarabo, Mancera & Afonso 2011; Herrera, Aragao, Hachero, Ruiz-Jarabo, Vargas-Chacoff, Mancera & Conceicao 2012). However, our experimental design did not consider these sampling points associated with acute stress situation, because it is focused in a long-term response. In this way, the highest cortisol values observed in this study was those obtained in the group of 28 hours of emersion (170.1 ± 21.1 ng/mL), being these values much lower than those obtained by other authors in stressed specimens of this species (Weber, 2009; Costas et al., 2011; Silva, Martins, Engrola, Marino, Averli & Conceicao 2010).

Plasma glucose values presented a similar trend than the previously reported in *S. maximus* specimens submitted to emersion (Calliens, 1996). In our case, glucose peak was ahead of cortisol enhancement, so that this energy substrate mobilization seems to be modulated by action of catecholamines, or even by a delay in the response to the cortisol hormone (Wendelaar Bonga, 1997). However, its values were reduced from 12 hours of emersion, indicating an increase in its consumption and/or decrease of hepatic free glucose release. Plasma lactate, another energy substrate used in stress situations (Mommsen, Vijayan & Moon 1999), presents a similar pattern of change that cortisol

and appears to be modulated by this hormone. Indeed, both parameters showed an increase over time, reflecting a stressful situation. Regarding with plasma triglycerides, proteins and amino acids, no marked variations were observed in relation to stress intensity. These results only highlighted the decline in the amino acids levels from 6 hours of emersion. This effect is probably associated with the conversion of these metabolites by the gluconeogenesis pathway to glucose (Mommensen et al., 1999), to maintain homeostasis.

In conclusion, we can establish a safe range of 28 hours for waterless transport in *S. senegalensis* without direct or delayed effect on mortality. Furthermore, in the absence of an in-depth analysis on the evolution of stress parameters between 0 and 2 hours emersion, it seems that this type of activity does not generate significant levels of stress previous to 6 hours of emersion. Nevertheless, it would be also desirable to study aspects such as i) the influence of anesthetics and other compounds on stress level attenuation, ii) the minimum size allowed for this transport system, or iii) the appropriate temperature to improve these results. In addition it will be necessary to have a good description of the baseline levels of stress markers in this species at different ages/weights, in order to determine when stress induced by waterless transport is really significant, as well as to determine the recovery time needed to return to the baseline levels.

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Figure legends

Figure 1. Time course changes in plasma (A) cortisol, (B) glucose and (C) lactate levels after different hours of emersion. Values are represented as mean $\pm$ S.E.M. Significant differences between sampling points are identified with different letters ($P < 0.05$, one-way ANOVA followed by Tukey's test).

Figure 2. Time course changes in plasma (A) triglycerides, (B) proteins and (C) amino acids levels after different hours of emersion. Values are represented as mean $\pm$ S.E.M. Significant differences between sampling points are identified with different letters ($P < 0.05$, one-way ANOVA followed by Tukey's test).

Tables

Table 1. Mortality rate during and after the emersion trial. The delayed mortality was evaluated during a period of four weeks.

<u>PARAMETERS</u>	<u>Time of emersion (hours)</u>							
	<u>0(Control)</u>	<u>2</u>	<u>6</u>	<u>12</u>	<u>20</u>	<u>28</u>	<u>36</u>	<u>44</u>
Mortality rate (%)	0	0	0	0	0	0	0	66.6
Delayed mortality rate (%)	0	0	0	0	14.3	0	42.8	16.7
Total mortality rate (%)	0	0	0	0	14.3	0	42.8	83.3

Table 2. Feeding performance of different experimental groups during a period of four weeks post-trial. Values represent the percentage of food eaten over the total food supplied. Different superscript letters represent significant differences between the means of each experimental group (p<0.05, Kruskal–Wallis analysis of variance).

<u>Time of emersion (Hours)</u>	<u>Days after emersion trial</u>																<u>Mean±SE</u>
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>15</u>	<u>16</u>	<u>17</u>	<u>18</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	
0(Control)	–	–	–	–	–	–	–	14,3	–	–	–	28,6	85,7	–	–	7,1	8,5 ± 5,5^{a,b}
2	–	–	–	–	28,6	28,6	42,9	21,4	42,9	–	28,6	14,3	92,9	100	78,6	85,7	35,3 ± 8,9^c
6	–	–	–	–	–	35,7	42,9	57,1	85,7	42,9	57,1	85,7	100	100	100	100	50,4 ± 10,3^c
12	–	–	–	–	–	35,7	–	42,9	85,7	7,1	–	71,4	71,4	100	100	100	38,4 ± 10,6^c
20	–	–	–	–	–	–	–	71,4	100	28,6	100	100	100	100	100	100	53,3 ± 12,2^c
28	–	–	–	–	–	–	–	–	28,6	–	28,6	28,6	66,7	100	41,7	100	26,3 ± 9,0^{a,c}
36	–	–	–	–	–	–	–	–	–	–	25	–	–	–	–	50	5,0 ± 3,5^{b,d}
44	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0,0 ± 0^d

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