Potential capacity of Senegalese sole (*Solea senegalensis*) to use carbohydrates: metabolic responses to hypo- and hyper-glycaemia

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Abstract

Glucose tolerance in fish is species-dependent and closely related to feeding habits. The capacity of each species to regulate glucose homeostasis is indicative of its potential to utilize dietary carbohydrates (CH). Senegalese sole is a carnivorous species but its nutrient requirements are still under debate. In the present study, we aimed to evaluate the capacity of Senegalese sole (20 ± 4 g) reared at 20°C to cope with marked changes in circulating levels of glucose through assessment of glucose tolerance by an oral glucose tolerance test and metabolic responses after induction of hyper-glycaemic and hypo-glycaemic conditions by intraperitoneal injections of D-glucose (600 mg.kg^{-1} body mass) or insulin (5 mg.kg^{-1} body mass), respectively. Changes observed in the levels of metabolites and enzyme activities at hepatic level show a metabolic response to counter-regulate acute changes in circulating glucose levels. Thus, glycogen levels in liver decreased markedly in hypo-glycaemic fish (16-fold change) and increased in hyper-glycaemic fish (1.3-fold change) compared with normo-glycaemic fish 24h after treatment. Furthermore, activities of G6Pase (involved in gluconeogenesis) and GPase (involved in glycogenolysis) were higher in hypo- than in hyper-glycaemic fish after 24h of treatment (2.9-fold change for G6Pase and 7.1-fold change for GPase). The present results indicate that Senegalese sole has a fast plasma clearance rate of glucose compared with other carnivorous fish species and show a good capacity to deal with different glycaemic conditions by undertaking metabolic changes able to restore glucose homeostasis. These results suggest that Senegalese sole may be able to use dietary CH efficiently, allowing the incorporation of more sustainable and cheaper energy sources in aquafeeds for this species.

Key words: glycaemia changes; insulin administration; glucose tolerance test; liver metabolism, gluconeogenesis, dietary CH utilization, Senegalese sole

1. Introduction

Glucose metabolism in fish has been addressed in numerous studies in the last years bringing to light some conclusive findings but many regulatory mechanisms still remain unclear (reviewed by Polakof et al., 2012). Glucose is not the preferential energy source for body functions in most fish species. However, this nutrient is important in fish since it is the principal fuel in some tissues like brain (Soengas and Aldegunde, 2002). As in diabetic mammals, most fish species were reported to be glucose-intolerant considering their post-prandial prolonged hyperglycaemia and low clearance rate of glucose in blood after inducing glucose loads by direct glucose administration or through feeding fish with diets containing high levels of carbohydrates (CH) (Moon, 2001). The degree of tolerance to glucose is different among fish species and seems to be related to their natural alimentary habits (Polakof et al., 2012). Thus, carnivorous species show lower tolerance to glucose than herbivorous or omnivorous species (Hemre et al., 2002, Stone, 2003; Enes et al., 2011).

Since glucose is the main monosaccharide obtained after CH digestion, oral glucose tolerance test (GTT) is commonly used as good indicator of CH utilization capacity (Stone, 2003). Results obtained for GTT performed in different fish species show that glucose clearance rate in fish is much lower than in mammals (Polakof et al., 2012). Since insulin production in fish is similar or even higher than in mammals glucose resistance in fish has been attributed to an inadequate regulation of glucose utilization (glycolysis) and production (gluconeogenesis) in fish liver (Enes et al., 2009). In this line, some studies reported an absence of inhibition of gluconeogenesis when plasma glucose levels were high in carnivorous species such as rainbow trout (Panserat et al., 2001) or gilthead sea bream (Caseras et al., 2000).

Senegalese sole (*Solea senegalensis*) is a good candidate for European aquaculture though its nutrient requirements have been only partially assessed. This species can digest and absorb dietary lipids (Borges et al., 2013a) but it cannot utilize or store them efficiently (Borges et al., 2013b). In these studies, fish fed with low lipid and high CH dietary levels showed a higher protein gain and increased growth (Borges et al., 2013b). Furthermore, it was recently reported that in Senegalese sole dietary lipids can compromise glucose homeostasis by affecting insulin signaling pathways in muscle (Borges et al., 2014). Other studies using different dietary CH:protein content ratios also suggest that Senegalese sole could utilize CH efficiently, based on growth performance and some changes in hepatic enzyme activities (Guerreiro et al., 2012). However, no studies are available about the metabolic response of

liver to different glycaemic conditions in Senegalese sole. Liver is the main tissue involved in the metabolic regulation of the response to changes in nutritional conditions (Enes et al., 2009). Therefore, the study of hepatic enzyme activity in response to induced hyper- and hypo-glycaemia reflects the capacity of fish to cope with different glucose levels. This information will serve to assess the potential capacity of this species to utilize dietary carbohydrates, and, therefore it would be useful to improve feed formulations.

Therefore, in the present study we aimed to evaluate the glucose tolerance of Senegalese sole by performing a GTT. The metabolic response to hypo- and hyper-glycaemia was characterized by evaluating metabolite levels in plasma (glucose) and liver (glucose and glycogen), and in the activity of key hepatic enzymes involved in pathways related to glucose metabolism such as glycolysis (Glucokinase-GK and Pyruvate kinase-PK), gluconeogenesis (Glucose-6-phosphatase-G6Pase, Fructose-1,6-biphosphatase-FBPase), glycogenolysis (Glycogen phosphorylase-GPase), and pentose phosphate shunt (Glucose-6-phosphate dehydrogenase-G6PDH). Some metabolites (triglycerides and total lipids) and enzyme activities (Hydroxyacyl-CoA dehydrogenase-HOAD) related to hepatic lipid metabolism were also evaluated in order to elucidate possible interactions between glucose and lipid metabolism.

2. Materials and methods

2.1 Fish

Senegalese sole (20 ± 4 g) were obtained from a commercial fish farm (Aquacria, Aveiro, Portugal). Fish were maintained for 2 weeks in quarantine in 400 liter tanks under a 12:12 L:D photoperiod, 25 ppt salinity, and 20 °C temperature. Fish were fed once daily (09:00 h) to satiety with commercial dry fish pellets (GEMMA DIAMOND 1.8 produced by SKRETTING; proximate composition was 62% crude protein, 9% carbohydrates, 16% crude fat, and 13% ash; 22 MJ.kg⁻¹ of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/EU), and were supervised by trained scientists (following the Federation of European Laboratory Animal Science Associations—FELASA category C recommendations).

2.2 Experimental protocol

2.2.1 Glucose tolerance test (GTT)

Senegalese sole fasted for 24 hours were anesthetized with MS-222 (200 mg.l⁻¹) and immediately weighed (mean weight 24.2 ± 1.3g) for oral administration of 10 ml.kg⁻¹ fish of a saline solution with 1g.kg⁻¹ of D-glucose. This concentration was selected according to previous studies in other fish species (Polakof et al., 2012). After oral administration, fish were placed in 7 individual tanks (one per sampling time). After 0.5, 1, 2, 4, 6, 10 and 24 hours, 10 fish were removed from holding tanks, anesthetized as above and blood sampled by caudal puncture with ammonium-heparinized syringes. Plasma samples were obtained after blood centrifugation, followed by deproteinization with 0.6 M perchloric acid and neutralization with 1 M potassium bicarbonate, frozen on dry ice, and stored at -80°C until further assay.

2.2.2 Glucose and insulin treatments

After acclimation, 10 fish (mean weight 26.5 ± 2.1g) per treatment and sampling time were fasted for 24 hours, lightly anesthetized with MS-222 (200 mg.l⁻¹), weighed and IP injected either with 10 µl.g⁻¹ body mass of saline solution alone (normo-glycaemic treatment) or with 5 mg bovine insulin.kg⁻¹ body mass (hypo-glycaemic treatment, , insulin from Sigma Chemical), or with 600 mg.kg⁻¹ body mass of D-glucose (hyper-glycaemic treatment). The doses used are in accordance with previous studies carried out in other fish species (Conde-Sieira et al., 2010). Furthermore, preliminary probes were performed in order to verify the suitability of these doses to induce hypo- or hyper- glycaemia in Senegalese sole. Immediately after injection, fish returned to individual tanks to be sampled 0.5, 1, 2, 4, 6, 10 and 24 hours later. On each sampling time, fish were removed from holding tanks, anesthetized as above, and weighed. Blood was collected by caudal puncture with ammonium-heparinized syringes. Plasma samples were obtained and processed as described above. Fish were sacrificed rapidly by decapitation and liver was removed, frozen in dry ice and stored at -80°C until assayed.

2.3 Assessment of metabolite levels and enzyme activities

Plasma glucose levels were quantified by using a commercial kit (Biomérieux) adapted to a microplate format. 50 mg of liver used for the assessment of metabolite levels were homogenized immediately by ultrasonic disruption with 7.5 vols of ice-cooled 0.6 M perchloric acid, and neutralized (using 1 M potassium bicarbonate). The homogenate was centrifuged, and the resulting supernatant was immediately frozen in dry ice and stored at -80°C until analysis. Liver glycogen levels were assessed using the method of Keppler and

Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Biomérieux, Spain). Triglyceride and NEFA levels were also analyzed with commercial kits (Spinreact and Wako, respectively).

Enzyme activities were assessed in 100 mg of liver samples homogenized by ultrasonic disruption with 9 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA; P-2714). The homogenate was centrifuged and the supernatant was immediately frozen on dry ice and stored at -80°C until analysis. Enzyme activities were determined in a microplate reader SPECTRAFluor (Tecan, Grödig, Austria) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (10-15 µl), at a pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µl), and allowing the reactions to proceed at 37 °C for pre-established times (3-15 min). Enzymatic analyses were carried out at maximum rates, with the reaction mixtures set up in preliminary tests to render optimal activities by adapting to Senegalese sole methods previously described for rainbow trout (Polakof et al., 2008). Enzyme activities were normalized by mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin (Sigma) as standard.

2.4 Statistics

In the glucose tolerance test, comparisons among groups were carried out using one-way ANOVA with time after glucose administration as main factor. In the glucose and insulin test, comparisons among groups were carried out using two-way ANOVA with time after treatment and glycaemic condition (normo-, hypo- and hyper-glycaemic) as main factors. When these tests showed significance ($P<0.05$), individual mean values were compared using Student-Newman-Keuls test. .

3. Results

3.1 Glucose tolerance test

Glucose levels in plasma after oral glucose administration are shown in Fig. 1. We observed a peak in glycaemia 1 hour after glucose administration, and at 2h values decreased 30% reaching levels similar to those of initials 10 hours after oral administration of glucose.

3.2 Metabolite changes under hypo- and hyper-glycaemic conditions

Glucose levels in plasma are shown in Fig. 2-A. Values in hyper-glycaemic fish were 200% higher than the normo-glycaemic fish 0.5 hours after glucose administration and peaked 4 hours later. 10 hours after glucose administration values decreased 50 %, and at 24 hours glucose recovered initial levels. Fish treated with insulin showed plasma glucose levels significantly lower than in normo-glycaemic group 10 hours after injection and at 24 hours glucose levels reached the lowest observed values. A comparison of the increase in glycemia (represented in %) between oral and ip administration is reflected in Fig. 2-B.

Changes in the levels of metabolites in liver are shown in Fig. 3. Glucose levels (fig. 3-A) in normo-glycaemic fish showed higher values than the other groups 10 hours after injection. In the group treated with glucose, values remained constant throughout sampling times whereas in hypo-glycaemic fish values peaked 1 hour after insulin administration and then decreased until showing significant lower levels than the other two experimental groups 24 hours after injection. Glycogen levels (Fig. 3-B) in normo-glycaemic group showed elevated levels at 1h compared with the other samplings and the other experimental groups. In hyper-glycaemic fish, glycogen levels were significantly higher than in the other samplings and other experimental groups 24 hours after injection. In contrast, the hypo-glycaemic group showed a progressive decrease in levels from the beginning to the end of the experimental times that were also lower than in the other groups. Levels of triglyceride (Fig. 3-C) did not significantly change throughout sampling times in normo-glycaemic fish whereas in hyper-glycaemic group they reached higher values from 10 to 24 hours after glucose administration compared with data at 0.5 h; values at 10h in hyper-glycaemic fish were different than the other experimental groups. Fatty acid levels in liver (Fig. 3-D) did not change throughout sampling times in normo-glycaemic fish whereas in the hyper-glycaemic group levels were higher than in the other glycaemic conditions 4 hours after glucose administration.

Enzyme activities in liver are shown in Fig. 4. GK activity (Fig. 4-A) did not show any difference with time within normo- and hypo-glycaemic groups whereas in hyper-glycaemic group a higher activity was observed after 24 hours compared with other sampling times. GK activity was also higher in hyper-glycaemic than other treated groups after 6 and 24 hours of glucose administration whereas lower activity was detected in hypo-glycaemic group 24 hours after insulin treatment. PK activity (Fig. 4-B) showed no significant differences with time in all treatments, although a lower activity was noted in the hypo-glycaemic group. GPase activity in liver (Fig. 4-C) peaked at 6h in normo-glycaemic group whereas in hyper-

glycaemic fish levels were lower compared to the other groups after 24 hours of glucose administration and significant higher values were observed in the hypo-glycaemic group 24h after treatment. G6Pase activity (Fig.5-A) showed no significant differences in normo-glycaemic group. Hyper-glycaemic group showed increased activity at 1 and 2h after glucose administration and from 2h to 24h values significantly decreased levels compared with the other groups. Hypoglycaemic group displayed lower values at 0.5 hours compared with those obtained at 2 and 6h. FBPase activity (Fig.5-B) showed higher values in fish 1h after insulin treatment and then recovered basal levels. No changes were noted for G6PDH activity (fig.5-C). The values obtained for HOAD activity (Fig.5-D) showed no significant differences in normo-glycaemic fish whereas in hyper-glycaemic fish higher values than in the other experimental groups were noted after 2h. In hypo-glycaemic fish, the activity of this enzyme was lower at 2h compared with the other sampling times.

4. Discussion

4.1 Tolerance to glucose in Senegalese sole

Oral glucose tolerance test is a common practice for the preliminary evaluation of glucose utilization capacity, and has been carried out in many fish species (Moon, 2001). Fish present a glucose clearance rate lower than that of mammals, which are able to recover glycaemic basal levels 3 hours after a glucose load (Polakof et al., 2012). However, this capacity is very variable among fish species, and relates to natural alimentary habits (Legate et al., 2001). Thus, omnivorous species such as tilapia, common carp or silver perch (Lin et al., 1995; Furuichi and Yone, 1981; Stone et al., 2003) show a faster glucose removal from the blood than carnivorous species like rainbow trout, Atlantic salmon or turbot (Palmer and Ryman, 1972; Hemre et al., 1995; García-Riera and Hemre, 1996). However, it should be taken into account that the omnivorous species selected in those studies are normally reared at higher temperatures than the carnivorous species that were considered for comparison; which could also affect glucose clearance rates observed. As far as we are aware, no previous studies described a GTT in Senegalese sole. There are some studies in this species evaluating the effect of different dietary CH compositions but focused on biometric parameters, nutrient utilization, and the activity of a few metabolic enzymes (Dias et al., 2004; Guerreiro et al., 2012). The present evaluation of GTT in Senegalese sole shows a 270% increase in plasma glucose levels 1 hour after administration, which decreased around 30% from 2 to 6 hours and recovered basal levels 10 hours after glucose administration. The time needed to reach the

maximum plasma level of glucose in Senegalese sole is lower than that observed in most carnivorous fish species studied so far, like rainbow trout, white sea bream, European sea bass or turbot (Palmer and Ryman, 1972; Enes et al., 2011; Enes et al., 2012; García-Riera and Hemre, 1996) using the same glucose dose and way of administration. However, this result is similar to that observed in omnivorous species such as common carp and channel catfish (Furuichi and Yone, 1981; Ng and Wilson, 1997). Although Senegalese sole mainly ingests invertebrates in the wild, and can then be considered as carnivorous, it has been reported that the digestive characteristics of this species, such as residual acid digestion and proteolysis in stomach and a rather long intestine, are more closely related to those generally found in fish with omnivorous feeding habits (Yúfera and Darías, 2007). GTT indicates the capacity to clear glucose loads from the blood and gives information about the potential capacity to utilize dietary CH (Moon 2001, Booth et al., 2006). In a previous study, Borges et al (2012b) suggested a preferential utilization of CH than lipids when dietary protein levels are lower than those normally required in this species. This putative capacity of Senegalese sole to utilize CH supports the GTT results showing a 30% clearance of plasma glucose load in 1 hour and 100% in 9h. As a whole, despite its carnivorous feeding habits, Senegalese sole is apparently perfectly able to remove glucose from plasma suggesting a high capacity to deal with dietary carbohydrates likewise omnivorous and/or herbivorous species.

4.2 Metabolic responses to glucose treatment

IP glucose treatment resulted in a plasma hyper-glycaemia similar to that observed after oral glucose treatment. However, several differences were observed between both responses, which could be attributed to glucose assimilation route. Although the plasma level of glucose doubled 1h after ip administration, this increase persisted until reaching the maximum value at 4h. Even so, total recovery was obtained at 10 hours, i.e. the same time needed after oral administration of glucose. The fact that glucose levels peaked faster after oral treatment compared with ip administration disagree with data obtained in other carnivorous fish species such as yellowtail kingfish (Booth et al., 2013), but is in agreement with previous studies carried out in the omnivorous common carp (Furuichi and Yone, 1981; Hertz et al., 1989). This is probably due to the peculiarities of Senegalese sole digestive system as previously mentioned, approaching this carnivorous species to herbivorous/omnivorous fish species. Furthermore, absolute values of glucose are higher when glucose was ip administered which can also be due to the dose used.

Liver is a key organ involved in glucose homeostasis. The regulation of glucose levels in blood depends on the balance between glucose utilization via glycolysis or glycogenesis and glucose production by gluconeogenesis or glycogenolysis in liver. An imbalance of this regulation could be the cause of glucose intolerance in fish (Enes et al., 2009). In the present study, we evaluated the activities of main enzymes and levels of metabolites involved in those pathways to characterize the metabolic potential of Senegalese sole to deal with different glycaemic conditions. No changes were observed in free glucose levels measured in liver in normo-glycaemic and hyper-glycaemic fish along the experiment. Since hepatic glucose levels are closely related to those of circulating glucose (due to the presence in liver of the high capacity glucose transporter GLUT 2), these results are indicative of a fast capacity of liver to buffer changes in glucose levels either through mobilization or through storage. Glycogen levels in hyper-glycaemic fish increased from 6h after glucose administration but these values were significantly higher only after 24 h. These results suggest that the deposition of glucose as hepatic glycogen in *Solea senegalensis* is slow and the extra available glucose from plasma could be used for other purposes. The increase in glycogen levels is similar to that described in other fish species after glucose administration (Conde-Sieira et al., 2011) or after feeding fish with carbohydrate-enriched diets (Enes et al. 2009, Hemre et al. 2002). Interestingly, our results also show that in normo-glycaemic group glycogen levels peaked 1h after saline injection, which could be due to the existence of circadian changes in this parameter. No daily rhythms of glycogen have been evaluated in Senegalese sole yet whereas it has been reported the absence of significant glucose rhythms in this species (Oliveira et al., 2013).

In all living organisms including fish, the main route for glucose catabolism is glycolysis (National Research Council (U.S.), 2011), which is induced by nutritional conditions. Thus, many fish species increased GK activity in liver under hyper-glycaemic conditions (Enes et al., 2006; Panserat et al., 2000; Tranulis et al., 1996; Conde-Sieira et al., 2011). Accordingly, in this study GK showed increased activities in hyper-glycaemic Senegalese sole 6 and 10 hours after glucose administration whereas hypo-glycaemic fish presented lower activities. Therefore, GK activities in Senegalese sole are correlated to glucose levels in plasma. Pyruvate kinase is another key enzyme involved in the glycolytic pathway but hepatic activity in fish is normally very low and poorly induced by nutritional conditions. The lack of response obtained in our study is in general in accordance with other previous results carried on in cod, plaice or rainbow trout (Enes et al, 2009).

Liver can obtain glucose through glycogenolysis (glycogen hydrolysis) or gluconeogenesis (endogenous production of glucose), but to maintain glucose homeostasis it is expected that these pathways are inhibited in liver under hyper-glycaemic conditions. GPase, the main enzyme involved in glycogen breakdown in fish liver (Enes et al., 2009), decreased its activity in hyper-glycaemic fish 24h after glucose administration, i.e. the time period when glycogen levels in liver were also significantly higher. Similar results were found in European perch that showed lower GPase activity after fed a 14% CH diet compared to fish fed a free-CH diet (Borrebaek and Christophersen, 2000).

One hypothesis used to explain the poor glucose tolerance in fish is the absence of inhibition of gluconeogenesis under hyper-glycaemic conditions (Panserat et al., 2002). Carnivorous fish, such as rainbow trout, European sea bass or gilthead sea bream fed with carbohydrates showed a poor inhibition of hepatic gluconeogenesis at both molecular and enzyme activity levels (Enes et al. 2006, 2008; Caseras et al., 2000). But Senegalese sole exhibited an inhibition of G6Pase activity 4 hours after glucose administration, which remained even 24h later. As far as we are aware, this is the first study in which a carnivorous fish species showed inhibited G6Pase activity under hyper-glycaemic conditions. These results are in agreement with the inhibition observed for G6Pase gene expression in trout and gilthead sea bream (Kamalam et al., 2013; Panserat et al., 2001, 2002) and the reduced G6Pase activity observed 16h after ingestion of dietary CH in Senegalese sole (Borges et al., 2014). Again, these results could be indicative of a better capacity of Senegalese sole to regulate glucose homeostasis compared with other carnivorous fish. However, no changes were found in the activity of the other gluconeogenic enzyme assessed (FBPase) in hyper-glycaemic fish, as observed previously in other carnivorous (rainbow trout) or omnivorous (common carp) fish species (Panserat et al., 2001, 2002) although inhibited FBPase activity by dietary CH was reported in gilthead sea bream (Panserat et al., 2002). Dietary lipid levels also affect gluconeogenesis in fish (Figueiredo-Silva et al., 2012), which should be considered in the studies where hyperglycemia is induced by dietary composition. In this line, it was also observed in Senegalese sole that dietary lipids compromise glucose homeostasis by affecting insulin signal pathways in muscle (Borges et al., 2014).

Glucose excess can be also converted into fatty acids by lipogenesis. Previous studies in rainbow trout indicated that lipogenesis is not induced by CH (Panserat et al., 2009) and seems to be regulated by insulin (Polakof et al., 2011). In this study, we found increased levels of triglyceride and FA in liver of hyper-glycaemic fish, which could be indicative that part of the glucose in excess is transformed into lipids. These results agree with Dias et al.

(2004) who also reported increments of liver lipid contents in Senegalese sole fed diets with high levels of CH. As for the activity of enzymes involved in lipid metabolism, we observed an increase in HOAD 4h after glucose administration, whereas no changes were observed in G6PDH. These results are slightly contradictory since under hyper-glycaemic conditions a decrease in lipolytic (HOAD) and an increase in lipogenic (G6PDH) capacities are expected.

4.3 Metabolic responses to insulin treatment

Since insulin treatment induced hypo-glycaemia, the changes described in parameters assessed can be attributed to the action of insulin alone or to the effects of hypoglycaemia induced by insulin. However, both mechanisms are suitable to elucidate the metabolic capacity of Senegalese sole to deal with changes in the levels of glucose. The effects of insulin treatment in fish liver metabolism have been evaluated through *in vitro* and *in vivo* studies in several species (reviewed by Polakof et al., 2012), but this is the first study in *Solea senegalensis*. A common response of fish treated with insulin is hypoglycemia and reduced hepatic glycogen contents (Polakof et al., 2012). In our study, glucose levels in plasma decreased from 2 hours after treatment onwards, and reached the lowest level 24 hours after injection. As a consequence, glycogen contents in liver also decreased dramatically. The enhanced glycogenolysis is partly reflected in the activity of GPase, which gradually increased from 4 hours after treatment onwards. Similar correlations between insulin treatment and GPase activities in liver were also found in *in vivo* studies with other fish species like common carp, red sea bream and yellowtail (Furuichi and Yone 1982a, b; Sugita et al., 1999). But in rainbow trout results vary depending according to *in vivo* and *in vitro* experiments (Pereira et al., 1995; Polakof et al., 2010a). Regarding insulin effects on gluconeogenesis, contradictory results have been obtained in fish describing activation (Navarro et al., 2006) and inhibition (Polakof et al., 2010b) of this pathway in liver. In our study, hypo-glycaemic fish showed higher G6Pase activity 2 and 6 hours after insulin administration compared with values registered at the initial and final sampling times. These results could be due to an enhancement of endogenous basal gluconeogenesis when circulating glucose levels decrease. This is further supported by an increased activity of FBPase (gluconeogenic enzyme) 2h after insulin administration. Regarding glycolytic parameters no changes were obtained for PK activity under insulin treatment whereas decreased activity was observed in GK, which agree with results obtained in other fish species (Plagnes-Juan et al., 2008; Polakof et al., 2010b). Moreover, no substantial effect of insulin was observed on lipid metabolism. Previous studies reported increased hepatic lipogenesis in

CH-fed rainbow trout stimulated by insulin (Polakof et al, 2011), but this effect was associated with the presence of circulating extra glucose which could not be observed in our study because of the lack of a combined glucose-insulin treatment.

As a whole the effects of insulin treatment (either directly or through induction of hypo-glycaemia) in liver metabolism of Senegalese sole such as enhanced glycogenolytic capacity or no changes in the gluconeogenic potential, seems to be more close to those described in omnivorous/herbivorous than in other carnivorous fish species.

5. Conclusions

In summary, this study evaluates for the first time, in Senegalese sole, parameters related to different glucose metabolism pathways to describe the metabolic response of this species to changes in glycaemic conditions. Glucose tolerance test indicate that Senegalese sole presents a fast clearance rate of glucose in plasma. Under hyper-glycaemia, extra glucose is moderately stored as glycogen or converted into lipids. Furthermore, endogenous production of glucose is inhibited when circulating glucose levels are high. On the other hand, insulin administration induces a prolonged hypoglycemia and marked loss of hepatic glycogen contents, but no effects were noticed in lipid metabolism of hypo-glycaemic fish.

In this context, the results obtained after hyper-glycaemic treatment are specially relevant for the aquaculture sector since they suggest that this species has a good capacity to cope with increased circulating levels of glucose that could have resulted from feeding carbohydrate-enriched diets. The potential capacity of Senegalese sole to utilize carbohydrates may help the aquafeed industry to consider dietary formulations based on more sustainable and cheaper feedstuffs. Nevertheless, the potential use of different carbohydrates sources requires further studies.

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Figure legends

Fig. 1. Glucose levels in plasma of Senegalese sole after oral administration of 10 ml.kg⁻¹ of saline solution with glucose (1g.kg⁻¹) sampled 0, 0.5, 1, 2, 4, 6, 10 and 24 hours after administration. Data represent mean $\pm$ SEM of 10 values. Different letters indicate significant differences ($P < 0.05$) among sampling times.

Fig. 2. A Glucose levels in plasma of Senegalese sole IP injected with 10 μ l.g⁻¹ body mass of saline alone (normo-glycaemic treatment) or with insulin (hypo-glycaemic treatment, 5 mg bovine insulin.kg⁻¹ body mass), or D-glucose (hyper-glycaemic treatment, 600 mg.kg⁻¹ body mass). Samples were taken at different times: 0, 0.5, 1, 2, 4, 6, 10 and 24 hours after treatments. Data represent mean $\pm$ SEM of 10 values. Different letters indicate significant differences ($P < 0.05$) among sampling times. * indicate significant differences ($P < 0.05$) compared with normo-glycaemic group. # indicate significant differences ($P < 0.05$) compared with hypo-glycaemic group. & indicate significant differences ($P < 0.05$) compared with hyper-glycaemic group. **B.** Glucose levels in plasma of Senegalese sole after oral or IP glucose administration (1g.kg⁻¹ or 600 mg.kg⁻¹) and sampled at different times: 0, 0.5, 1, 2, 4, 6, 10 and 24 hours after glucose administration. Data represent mean $\pm$ SEM of 10 values.

Fig. 3. Glucose (**A**), glycogen (**B**), triglyceride (**C**) and fatty acid (**D**) levels in liver of Senegalese sole IP injected with 10 μ l.g⁻¹ body mass of saline alone (normo-glycaemic treatment) or with insulin (hypo-glycaemic treatment, 5 mg bovine insulin.kg⁻¹ body mass), or D-glucose (hyper-glycaemic treatment, 600 mg.kg⁻¹ body mass). Samples were taken at different times: 0, 0.5, 1, 2, 4, 6, 10 and 24 hours after treatments. Data represent mean $\pm$ SEM of 10 values. Data represent mean + SEM of 10 measurements. Further details as in legend to Figure 2.

Fig. 4. Activities of glucokinase-GK (**A**), pyruvate kinase-PK (**B**) and glycogen phosphorylase-GPase (**C**) in liver of Senegalese sole IP injected with 10 μ l.g⁻¹ body mass of saline alone (normo-glycaemic treatment) or with insulin (hypo-glycaemic treatment, 5 mg bovine insulin.kg⁻¹ body mass), or D-glucose (hyper-glycaemic treatment, 600 mg.kg⁻¹ body mass). Samples were taken at different times: 0, 0.5, 1, 2, 4, 6, 10 and 24 hours after treatments. Data represent mean $\pm$ SEM of 10 values. Data represent mean + SEM of 10 measurements. Further details as in legend to Figure 2.

Fig. 5. Activities of Glucose-6-phosphatase-G6Pase (**A**), Fructose-1,6-biphosphatase-FBPase (**B**) and Glucose-6-phosphate dehydrogenase-G6PDH (**C**) and Hydroxyacyl-CoA dehydrogenase-HOAD (**D**) in liver of Senegalese sole IP injected with 10 μ l.g⁻¹ body mass of saline alone (normo-glycaemic treatment) or with insulin (hypo-glycaemic treatment, 5 mg bovine insulin.kg⁻¹ body mass), or D-glucose (hyper-glycaemic treatment, 600 mg.kg⁻¹ body mass). Samples were taken at different times: 0, 0.5, 1, 2, 4, 6, 10 and 24 hours after treatments. Data represent mean $\pm$ SEM of 10 values. Data represent mean + SEM of 10 measurements. Further details as in legend to Figure 2.

Fig. 1

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