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# Insulin-induced hypoglycaemia is co-ordinately regulated by liver and muscle during acute and chronic insulin stimulation in rainbow trout (*Oncorhynchus mykiss*)

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## SUMMARY

**The relative glucose intolerance of carnivorous fish species is often proposed to be a result of poor peripheral insulin action or possibly insulin resistance. In the present study, data from aortic cannulated rainbow trout receiving bovine insulin (75 mIU kg$^{-1}$) injections show for the first time their ability to clear glucose in a very efficient manner. In another set of experiments, mRNA transcripts and protein phosphorylation status of proteins controlling glycaemia and glucose-related metabolism were studied during both acute and chronic treatment with bovine insulin. Our results show that fasted rainbow trout are well adapted at the molecular level to respond to increases in circulating insulin levels, and that this hormone is able to potentially improve glucose distribution and uptake by peripheral tissues. After acute insulin administration we found that to counter-regulate the insulin-induced hypoglycaemia, trout metabolism is strongly modified. This short-term, efficient response to hypoglycaemia includes a rapid, coordinated response involving the reorganization of muscle and liver metabolism. During chronic insulin treatment some of the functions traditionally attributed to insulin actions in mammals were observed, including increased mRNA levels of glucose transporters and glycogen storage (primarily in the muscle) as well as decreased mRNA levels of enzymes involved in *de novo* glucose production (in the liver). Finally, we show that the rainbow trout demonstrates most of the classic metabolic adjustments employed by mammals to efficiently utilize glucose in the appropriate insulin context.**

Key words: fish, insulin, glucose metabolism, liver, muscle.

## INTRODUCTION

Carnivorous fish species like rainbow trout (*Oncorhynchus mykiss*) are traditionally considered as glucose intolerant (Moon, 2001; Wilson, 1994) due primarily to the prolonged hyperglycaemia experienced after a glucose load or intake of carbohydrate-enriched meals (Bergot, 1979; Palmer and Ryman, 1972). Although initially the basis for this poor metabolic glucose utilization was though to be a deficiency in insulin secretion (Furuichi and Yone, 1981; Palmer and Ryman, 1972), later hormone titration in several fish species demonstrated that plasma insulin levels in piscine species are even higher than in mammals (Mommsen and Plisetskaya, 1991). The secretagogues of insulin in fish are the same as in mammals, and although glucose is not the most potent of these, generally hyperglycaemia does result in hyperinsulinaemia (Moon, 2001). To exert its biological effects, insulin binds to a specific transmembrane receptor that possesses tyrosine kinase activity (Gutiérrez et al., 2006). Moreover, insulin is also able to induce Akt (critical node in the insulin signalling pathway in mammals) (Taniguchi et al., 2006) phosphorylation *in vivo* and *in vitro* in muscle and liver of rainbow trout (Castillo et al., 2006; Plagnes-Juan et al., 2008; Seiliez et al., 2008a).

Insulin functions primarily as a major anabolic hormone by stimulating postprandial glucose uptake by the liver and skeletal muscle, depressing rates of hepatic gluconeogenesis, and activating glycogen synthesis and lipogenesis. In fish, the most prominent response to exogenous insulin injection is hypoglycaemia (Ince, 1983b). However, both the magnitude and duration of this hypoglycaemic effect are dependent on several factors, including insulin type and dose level, route of injection, season, nutritional state and previous nutritional history (Ince, 1983b; Mommsen and Plisetskaya, 1991).

The mechanism by which insulin regulates plasma glucose levels in fish remains unknown, and the relative contribution of the main peripheral tissues sensitive to this hormone remain to be clarified (Navarro et al., 2006). Regarding glucose uptake, in addition to GLUT4 (glucose transporter type 4) translocation to the plasma membrane in response to insulin in fish skeletal muscle (Díaz et al., 2007), both GLUT1 (glucose transporter type 1) and GLUT4 gene expression are regulated by this hormone *in vivo* and *in vitro* (Capilla et al., 2002; Díaz et al., 2009). On the other hand, although insulin administration often results in unspecific glycogen fluctuations (Mommsen and Plisetskaya, 1991), the most common response is the depletion of hepatic glycogen stores *in vivo* (Bhatt et al., 1980; Ottolenghi et al., 1982), while a more consistent glycogenic response is found *in vitro* (Foster and Moon, 1990; Ottolenghi et al., 1981). The response of muscle glycogen to insulin administration seems to be more consistent, as in several fish species muscle glycogen levels increase following physiological hormone injection (Bhatt et al., 1980; Ottolenghi et al., 1982). Concurrent with its action on glucose uptake, insulin depresses the rate of gluconeogenesis in some salmonids (De la Higuera and Cárdenas, 1986), but not in other fish species (Mommsen and Plisetskaya, 1991). Consistent with these observations, insulin is also able to down-regulate *in vivo* and *in*

*vitro* the expression of the main hepatic gluconeogenic genes in rainbow trout (Plagnes-Juan et al., 2008).

As previously stated by other authors (Mommsen and Plisetskaya, 1991; Navarro et al., 2006), most studies involving insulin in fish are performed using pharmacological doses of the hormone, and thus these data need to be interpreted carefully. Studies employing physiological doses of insulin are scarce (Capilla et al., 2002; Ince, 1983a; Ince and Thorpe, 1976), and as far as we are aware, in only one of these was the plasma insulin level measured after exogenous administration (Pérez-Sánchez, 1988). On the other hand, almost all the studies carried out to date have focused on acute insulin effects, subjecting fish to transient levels of the hormone, and only a few involved chronic trials (Ablett et al., 1981; Polakof et al., 2008a; Slagter et al., 2005). Therefore, the aim of the present study utilizing the carnivorous rainbow trout was 2-fold: (i) to better understand the capacity of insulin to improve glucose clearance and peripheral utilization using aortic cannulated trout; and (ii) to investigate the metabolic effects of insulin by employing physiological doses during both acute intraperitoneal injection and chronic mini-pump implantation treatments. As well, for the first time the two main insulin targets, skeletal muscle and liver, were studied at both the biochemical and molecular level to analyse the cellular origins responsible for the plasma glucose profiles observed.

## MATERIALS AND METHODS

### Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from the INRA experimental fish farm facilities at Donzacq (Landes, France). Fish were maintained in open circuit tanks supplied with 17°C well-aerated water under a controlled photoperiod (12 h:12 h L:D) and fed with a commercial diet (T-3P classic, Trouw, Vervins, France). Fish mass for the cannulation experiments was 350±9 g (female), and for the other trials it was 170±4 g (immature). The experiments were conducted following the Guidelines of the National Legislation on Animal Care of the French Ministry of Research.

### Experimental protocols

Fish dorsal aorta cannulation procedures were performed according to Zohar (Zohar, 1980), allowing recovery from surgery for 24 h under free-swimming conditions. Trout were fasted for 48 h and then subjected to four different experiments involving injection of solutions through the cannula into the dorsal aorta: (i) glucose injection (250 mg $kg^{-1}$), (ii) glucose plus bovine insulin (75 mIU $kg^{-1}$ or ~2.7 μg $kg^{-1}$, Sigma, St Louis, MO, USA) injection, (iii) glucose injection 15 min (peak of glucose in plasma) after the first glucose administration, and (iv) glucose plus insulin injection 15 min after the first glucose administration. The insulin dose was specifically chosen to lie within the range commonly used in other fish and mammalian studies (between 50 and 120 mIU $kg^{-1}$) (DeFronzo et al., 1979; Ince, 1982). Blood was sampled *via* the cannula at different time points after treatment administration: 0, 15, 30, 45, 60, 90 and 120 min. Groups of three fish were sampled at each time point. After each sampling, blood was flushed back into the cannula and the cannula filled with heparin saline solution, which was discarded at each sampling time. Blood was immediately centrifuged and analysed for glucose and bovine insulin levels (see below). The *K* coefficient was calculated following the modified formula suggested by Ottolenghi and colleagues (Ottolenghi et al., 1995):

$$K = \frac{\log C_1 - \log C_2}{\log(t_2 - t_1)} ,$$

where *C* is the insulin concentration and *t* the time.

Acute insulin administration was done using 48 h-fasted fish that were lightly anaesthetized with 0.05% (v/v) 2-phenoxyethanol, weighed, and intraperitoneally injected with 5 ml $kg^{-1}$ body mass of saline solution alone (control, *N*=8) or saline containing bovine insulin at 1.4 IU $kg^{-1}$ (or ~52 μg $kg^{-1}$) body mass (*N*=8). Sampling was initiated 6 h after injection. Eight fish per group were randomly killed by cervical section and sampled. Blood was removed from the caudal vein into ammonium-heparinized syringes and centrifuged (3000 ***g***, 5 min), and the recovered plasma was immediately frozen and kept at –20°C pending analyses. Liver and a sample of dorso-anterior white muscle were dissected and immediately frozen in liquid nitrogen and kept at –80°C pending analyses.

For chronic insulin administration, we used trout that were food deprived for 48 h and then implanted with 1003D Alzet® mini-osmotic pumps (Durect Corp, Cupertino, CA, USA) containing either saline solution (control, *N*=8) or bovine insulin solution (Sigma, *N*=8). Mini-pump flow rate was established to be 0.39 μl $h^{-1}$, which at 17°C should provide sustained release of insulin at 0.35 IU $kg^{-1}$ $day^{-1}$ (or 13 μg insulin $kg^{-1}$ $day^{-1}$) for 11 days. Fish were first anaesthetized and their body mass determined. Mini-pumps were inserted into the peritoneal cavity through a 1.0 cm incision made in the ventral midline at *ca*. 2.0 cm rostral of the pelvic fins. The incision was closed with one stitch and antibiotic ointment was applied topically to the incision area. After 4 days, eight fish per group were killed and sampled as above.

### Molecular and biochemical analysis

Gene expression levels were determined by real-time quantitative RT-PCR as previously (Díaz et al., 2009; Kolditz et al., 2008; Plagnes-Juan et al., 2008). Primers were designed to overlap an intron if possible (Primer3 software) using known sequences in nucleotide databases (GenBank and INRA-Sigenae), except for 6-phosphofructo-1-kinase, liver isoform (6PF1K-L; forward primer: GGTGGAGATGCACAAGGAAT; reverse primer: CTTGATGTTGTCCCCTCCAT; tcbk0069c.k.05_s.1) and 6-phosphofructo-1-kinase, muscle isoform (6PF1K-M; forward primer: GGGACCTCGAGATGAACGTA; reverse primer: GAGGGCGAAAGATGAAGTCTG; tcad0007a.e.10_3.1.2.1). Quantification of the target gene transcript was done using *ef1α* gene expression as the reference (Olsvik et al., 2005), which was stably expressed in this experiment. Relative quantification of the target gene transcript with the *ef1α* reference gene transcript was done following the Pfaffl method (Pfaffl, 2001).

Protein extraction (20 μg of protein for liver and muscle) and western blotting were developed as described elsewhere (Plagnes-Juan et al., 2008) using anti-phospho-Akt Ser473, anti-Akt and anti-β-tubulin antibodies (Cell Signaling Technology, Ozyme, St Quentin-en-Yvelines, France). These antibodies were shown to successfully cross-react with rainbow trout Akt and β-tubulin proteins (Seiliez et al., 2008b).

Plasma glucose levels were determined using a commercial kit (Biomérieux, Marcy l'Etoile, France) adapted to a microplate format. Bovine insulin levels were measured using a commercial ELISA kit (Mercodia, Uppsala, Sweden). Since exogenous bovine and not endogenous trout insulin [including two isoforms whose biological effectiveness remains to be clarified (Plisetskaya, 1989) but is expected to be extremely low in 6 day-fasted fish (see Navarro et al., 2006)] levels were determined, we cannot disregard possible mixed effects between the two insulin types in the present study. Glycogen levels [following the method of Keppler et al. (Keppler et al., 1974)] and enzyme activities were determined as described previously (Polakof et al., 2008b). Briefly, glycogen phosphorylase (GPase) was

assayed using 50 mmol $l^{-1}$ phosphate buffer (pH 7.0), 27 mmol $l^{-1}$ $MgSO_4$, 19.5 mmol $l^{-1}$ EDTA, 0.5 mmol $l^{-1}$ $NADP^+$, 5 mmol $l^{-1}$ glucose 1,6-bisphosphate, 2.5 mmol $l^{-1}$ AMP, excess phosphoglucomutase, excess glucose 6-phosphate dehydrogenase and 10 mg $ml^{-1}$ glycogen (omitted in controls). GPase *a* activity was measured with 10 mmol $l^{-1}$ caffeine present and total GPase activity was estimated without caffeine. The ratio of GPase activity with and without caffeine multiplied by 100 represents the percentage of total GPase (*a*+*b*) in the active form (% GPase *a*). Glycogen synthetase (GSase) was assessed using 50 mmol $l^{-1}$ imidazole-HCl (pH 7.5), 150 mmol $l^{-1}$ KCl, 15 mmol $l^{-1}$ $MgCl_2$, 5 mmol $l^{-1}$ phosphoenolpyruvate, 0.15 mmol $l^{-1}$ NADH, 2 mg $ml^{-1}$ glycogen, excess pyruvate kinase, excess lactate dehydrogenase and 6 mmol $l^{-1}$ UDP-glucose (omitted in controls). Total GSase activity was measured with 5 mmol $l^{-1}$ glucose 6-phosphate present, and GSase *b* activity was estimated in the absence of glucose 6-phosphate. The ratio of GSase activity without and with glucose 6-phosphate represents the percentage of total GSase (*a*+*b*) in the active form (% GSase *a*).

### Statistical analysis

Data are means ± s.e.m. Comparisons among groups and times for aortic administrations were performed using a two-way ANOVA (SigmaStat; SPSS, Chicago, IL, USA) with treatments (glucose, insulin, glucose plus glucose or glucose plus insulin) and time (0, 15, 30, 45, 60, 90 and 120 min) as independent variables. *Post hoc* comparisons were made using a Student–Newman–Keuls test, and differences were considered statistically significant at $P<0.05$. Results of intraperitoneal injection and implantation of insulin administration are expressed as means ± s.e.m. ($N$=8) and were analysed by one-way ANOVA using Student–Newman–Keuls multiple comparison test *a posteriori*. The level of significance was set at $P<0.05$. When necessary (ratios and data normalization), data were log-transformed to fulfil the conditions of the analysis of variance.

## RESULTS

### Plasma glycaemia in cannulated fish

Plasma glucose levels in aortic cannulated trout are shown in Fig. 1. Plasma glycaemia reached its maximum level 15 min after a single glucose injection into the dorsal aorta, returning slowly towards the basal level over the next 2 h, and recovering completely 8 h after the treatment (data not shown). The same time course profile was found after the co-administration of glucose and insulin, although the maximum glycaemic levels were lower than after the administration of glucose alone. In contrast to this single injection, the administration of glucose 15 min after a first glucose injection produced a continuous increase in glycaemia that was, however, 4-fold lower than the first one. The maximum glucose levels were reached 30 min after the first injection. This observation was also supported by the $K$ coefficients, as the $K_{(30–120)}$ value for the single glucose injection was 0.17% $min^{-1}$, while after the double glucose injection the coefficient reached 0.22% $min^{-1}$. Similar to the profiles obtained in the first treatments, the co-administration of glucose and insulin after a first glucose injection produced a similar kinetic glucose appearance, but showed a lower maximum level. In this case, the relative increase after the second injection was 3.2-fold smaller than after the first administration.

### Plasma glycaemia after acute and chronic insulin treatment

After both intraperitoneal injection and implantation treatment of insulin, glucose levels in 48 h-fasted trout decreased significantly ($P<0.05$) with respect to their controls (Fig. 2A). In these treatments, hyperinsulinaemia was achieved 6 h (acute) and 4 days (chronic) after insulin administration (Fig. 2B). For the acute administration, the insulin dose was chosen based on preliminary studies (data not shown) in which doses commonly used in fish (10–60 IU $kg^{-1}$) demonstrated insulin levels 6 h after injection that were 20-fold higher than the normal range described in fish (Mommsen and Plisetskaya, 1991). Based on the results of Inui and Gorbman (Inui and Gorbman, 1977) and the recommendation of Mommsen and Plisetskaya (Mommsen and Plisetskaya, 1991) to achieve physiological insulin doses, fish were injected with 1.4 IU $kg^{-1}$ of bovine insulin, achieving ~30 ng $ml^{-1}$ of plasma insulin and significant hypoglycaemia. For the chronic treatment the insulin dose was chosen based on previous trials carried out by us in order to determine the optimal concentration to achieve hypoglycaemia in fish in that period of time, as recommended by Mommsen and Plisetskaya (Mommsen and Plisetskaya, 1991). In this case, after 4 days of treatment insulin levels in trout were 1.52±0.23 ng $ml^{-1}$; higher doses were found to cause symptoms of irreversible coma and death in fasted fish (usually glycaemia <1–1.5 mmol $l^{-1}$) while the dose we used here produced a mild hypoglycaemia in healthy animals.

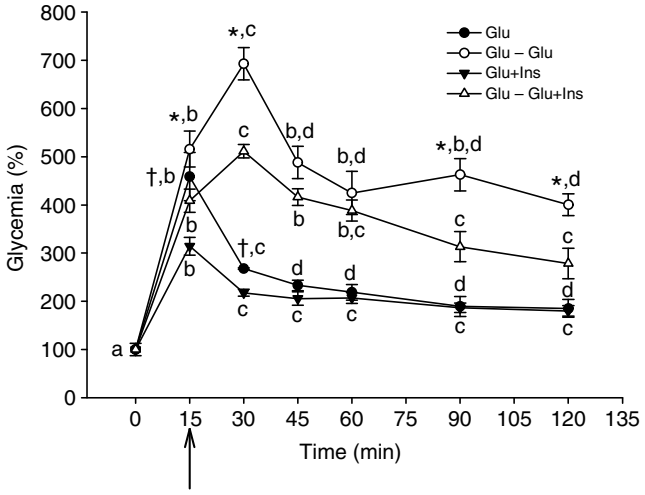


Fig. 1. Time course of plasma glucose levels in rainbow trout after aortic injection of glucose alone (250 mg $kg^{-1}$), glucose plus bovine insulin (75 mIU $kg^{-1}$), glucose alone 15 min after a first glucose administration, and glucose plus insulin 15 min after a first glucose administration. Results are expressed as means ± s.e.m. ($N$=3) and are represented as a percentage with respect to the preinjection glucose levels in each group. Mean initial plasma glycaemia (100%) was 4.35±0.39 mmol $l^{-1}$. Comparisons were made by two-way ANOVA followed by Student–Newman–Keuls multiple comparison test. *Significant difference between glucose plus glucose and glucose plus glucose and insulin injected fish at each sampling time ($P<0.05$). †Significant difference between glucose and glucose plus insulin injected fish at each sampling time ($P<0.05$). Different letters indicate significant differences between each sampling time within each injected group. The arrow indicates the time of the second injection.

### Insulin signalling pathway after acute and chronic insulin treatment

Total Akt protein levels and Akt phosphorylation status in liver and muscle are presented in Fig. 3. After both acute and chronic insulin administration, Akt phosphorylation was increased 2- to 3-fold in liver, although no changes were noted in the total content of this protein. In skeletal muscle, total Akt and phosphorylated Akt

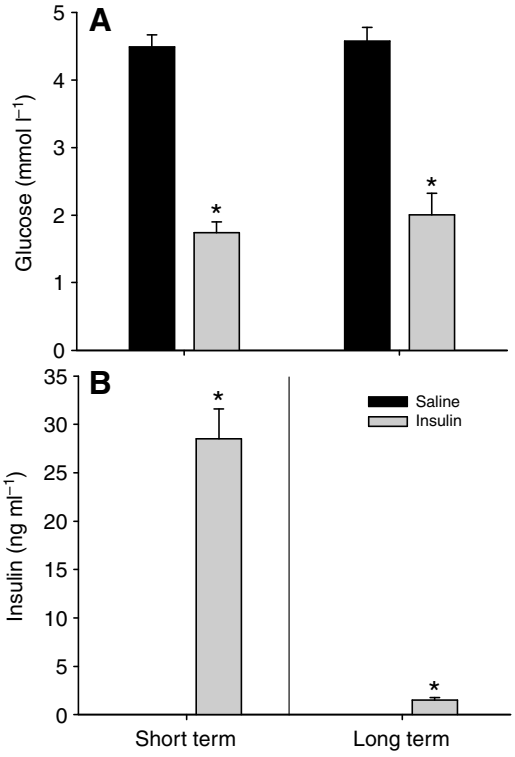


Fig. 2. Plasma glucose (A) and bovine insulin (B) levels 6 h after intraperitoneal administration of insulin (1.4 IU kg$^{-1}$, short term) or after 4 days of insulin implantation (0.35 IU kg$^{-1}$, long term) in 48 h-fasted rainbow trout. Control groups received, respectively, saline injection or implantation. Results are expressed as means ± s.e.m. ($N$=8) and were analysed by one-way ANOVA followed by Student–Newman–Keuls multiple comparison test. *Significant difference between insulin- and saline-treated fish ($P$<0.05).

increased 1.75- and 4-fold, respectively, in fish receiving a single hormone injection (the ratio of p-Akt to total Akt did increase in insulin-treated trout); however, none of the Akt forms were affected by the chronic hormone infusion in muscle.

### Insulin-related gene expression after acute and chronic insulin treatment

Changes in relative mRNA transcripts of key glycolytic and gluconeogenic enzymes are shown in Fig. 4. Transcript levels of glucose transporter type 2 (GLUT2; Fig. 4A) and hexokinase (HK; Fig. 4B) over the short term were up-regulated in insulin-injected fish, although transcript levels of other glycolytic enzymes remained unaffected by the hormone. In contrast, glucokinase (GK) mRNA levels (Fig. 4C) were diminished by insulin, resulting in a 100-fold decrease in the levels of this enzyme. Transcript levels of glucose 6-phosphatase (G6Pase; Fig. 4F) were greatly increased by insulin, as were those of fructose 1,6-biphosphatase (FBPase; Fig. 4G). Phosphoenolpyruvate carboxykinase (PEPCK) transcript levels (Fig. 4H) were unaffected by insulin administration. Long-term administration of insulin, however, resulted in only minor changes in transcript levels of glycolytic enzymes like pyruvate kinase (PK; Fig. 4D), except for GK, which was strongly inhibited by insulin.

Relative transcript levels of key glycolytic enzymes and glucose transporters in muscle are presented in Fig. 5. In muscle, acute insulin treatment elicited a common down-regulation response in levels of GLUT1 (Fig. 5A) and GLUT4 (Fig. 5B) transporters, and the glycolytic enzymes HK (Fig. 5C) and PK (Fig. 5D), while 6PF1K (Fig. 5E) levels were unaffected by insulin injection. No changes in transcript levels of the glycolytic enzymes were found under long-term insulin treatment. However, expression of both glucose transporters was stimulated by chronic administration of the hormone.

### Glycogen metabolism after acute and chronic insulin treatment

Glycogen levels, GPase and GSase enzyme activities in liver and white skeletal muscle of rainbow trout are shown in Fig. 6. Muscle glycogen levels increased dramatically up to 7-fold in insulin-injected fish. This increase was paralleled by increases in both total and activated GSase activity, while no changes were noted in total GPase or percentage GPase *a* activity. In contrast, long-term insulin administration was characterized by a 3-fold increase in glycogen levels but no changes in enzymes regulating its synthesis or depletion. In liver, short-term insulin administration produced a significant decrease in glycogen levels, which was followed by increases in both total GPase and percentage GPase *a* activity; no changes occurred in GSase activity. Long-term insulin infusion also decreased glycogen levels, but as in muscle, glycogen-regulating enzymes were not affected by the hormone.

## DISCUSSION

The major anabolic functions of insulin action in fish are focused in the liver and skeletal muscle (Mommsen and Plisetskaya, 1991). Moreover, other classical functions attributed to insulin in mammals, including increases in hepatic glycogen stores and inhibition of gluconeogenesis, are not always observed in fish, and opposite or lack of actions are often described (Navarro et al., 2006). Although hypoglycaemia is the most robust effect described in fish after insulin administration (Mommsen and Plisetskaya, 1991), the mechanism by which this happens remains under debate and the contribution of the different peripheral tissues remains to be elucidated. In the present study we utilized aortic cannulation to elucidate the ability of insulin to promote glucose clearance and peripheral utilization in trout, as well as acute and chronic insulin administration to study the metabolic effects of this hormone at both biochemical and molecular levels.

### Plasma glycaemia regulation by insulin in cannulated trout

While the physiological actions of insulin and its plasma kinetic dynamics have been studied in fish (Ince, 1982; Navarro et al., 2006), its ability to induce glucose clearance has received little attention (Thorpe and Ince, 1974). In the present study, we found that if a second glucose bolus is administrated to fasted trout, the glycaemic increase is 50% lower than after a single injection. This observation is also supported by the increased $K$ coefficient following this second injection. However, it must be noted that even here the $K$ coefficient is still 9- to 20-fold lower than that of mammals (Appleton et al., 2001; Yajima et al., 1983) and 25-fold higher than that of omnivorous fish species (Ottolenghi et al., 1995) but similar to those of other carnivorous fish (Ince and Thorpe, 1974). Thus, in agreement with data from other fish species (Ince and Thorpe, 1974), these results suggest that rainbow trout can develop an improved capacity to clear and peripherally utilize glucose after the first injection and will be more efficient at dealing with the second load. This improvement in glucose clearance efficiency after a first glucose load probably reflects an insulin-assistance process, including stimulation of glucose-metabolizing enzyme activities and gene expression. We also performed, for the first time in trout, co-

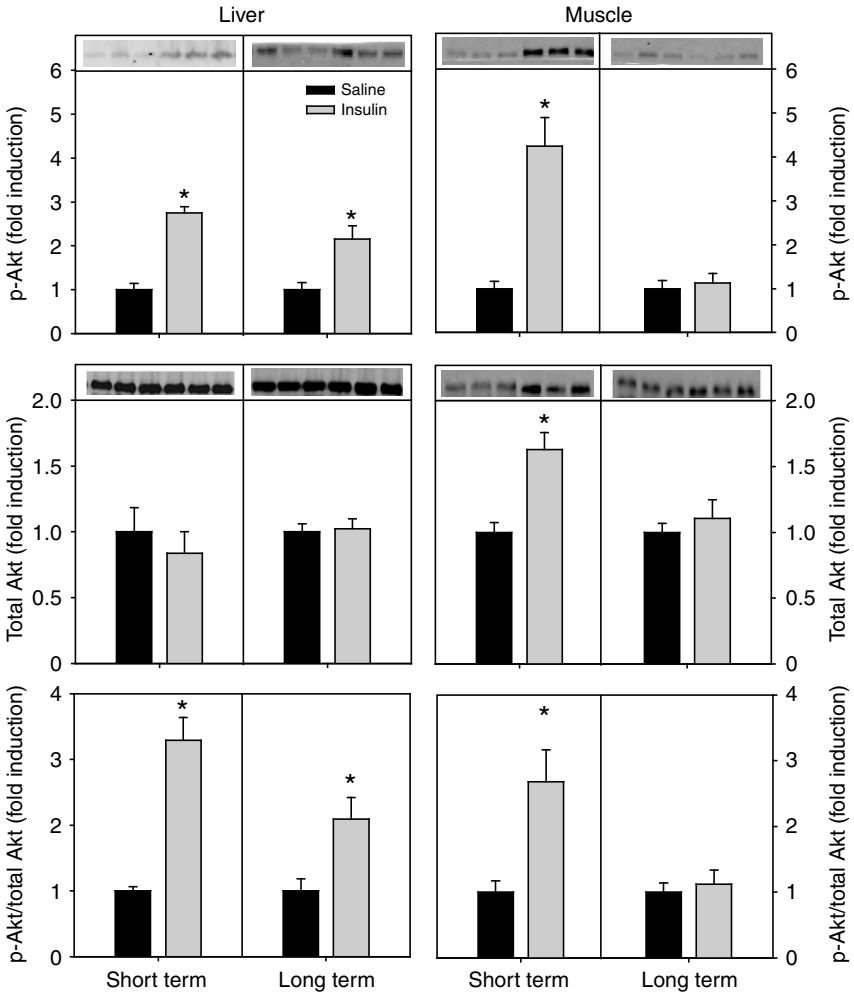


Fig. 3. Western blot analysis of Akt phosphorylation (p-Akt), total Akt protein, and p-Akt to total Akt ratio in rainbow trout liver and muscle after intraperitoneal administration of insulin (6 h) or insulin implantation (4 days). The gel was loaded with 20 μg of total protein per lane. Protein and phosphorylation levels were normalized with β-tubulin levels and are indicated as fold variation of the saline solution-treated group. Results are expressed as means ± s.e.m. (*N*=8) and were analysed by one-way ANOVA followed by Student–Newman–Keuls comparison test. *Significant difference (*P*<0.05). For more details see Fig. 2.

injections of glucose and insulin. The results obtained are in agreement with those mentioned above regarding double injection of glucose, in which lower maximum values of glycaemia and faster glucose clearances were found. These results are in agreement with a similar trial carried out in heart-cannulated pikes (*Esox lucius*) (Thorpe and Ince, 1974), in which co-injection of insulin and glucose resulted in a greatly improved glucose tolerance, as observed by us in rainbow trout. Together, these results indicate that in trout exogenous insulin can improve glucose distribution and uptake by peripheral tissues.

### Acute insulin administration: metabolic regulation in muscle and liver

Most of the information concerning insulin action on fish metabolism has been provided by intraperitoneal administration of the hormone (Mommsen and Plisetskaya, 1991). However, the dose chosen for such studies was often pharmacological (Ince, 1983b; Mommsen and Plisetskaya, 1991; Navarro et al., 2006; Plagnes-Juan et al., 2008) and the information collected, while valid, must be interpreted carefully. In the present study trout were intraperitoneally injected with insulin at 1.4 IU $kg^{-1}$, and the moderate hypoglycaemia (~2 mmol $l^{-1}$) and insulin levels [~30 ng $ml^{-1}$, within the range observed post-prandially in this species (Mommsen and Plisetskaya, 1991)] observed in the trout 6 h after the hormone administration allow us to interpret our data from a physiological point of view.

The most probable scenario explaining hypoglycaemia in insulin-injected rainbow trout in the present study is the concomitant and dramatic increase in muscle glycogen levels. This increased storage of glucose as glycogen after insulin administration was previously reported in several fish species (Bhatt et al., 1980; Emdin, 1982; Ottolenghi et al., 1982) and could be related to the changes in Akt protein found in skeletal muscle. After insulin injection we detected not only more active (phosphorylated) Akt but also more total Akt protein, probably resulting in a greater magnitude of insulin-stimulated signal (Gosmanov et al., 2004), allowing a faster and stronger response of the muscle to the hormone stimulation. Due to the high relative mass of the white skeletal muscle in fish, a rapid response in acute insulin stimulation could trigger a rapid and large glucose uptake and glycogen storage, explaining the acute hypoglycaemia experienced by insulin-injected trout. This effect also matches the classical action of insulin in mammals, in which stimulation of glucose uptake by GLUT4 and activation of GSase (through phosphorylation of Akt) are involved (Yeaman et al., 2001). However, in contrast to other studies in trout (Capilla et al., 2002; Díaz et al., 2009) no increases in either GLUT1 or GLUT4 transcript levels were found during hyperinsulinaemia; rather, a down-regulation was observed. Since GLUT4 is mainly regulated by translocation to the membrane (Díaz et al., 2007), changes in glucose uptake by this regulatory process cannot be ruled out in the present study. Nevertheless, our results in hypoglycaemic trout are consistent with the response observed in mammals, where skeletal muscle is

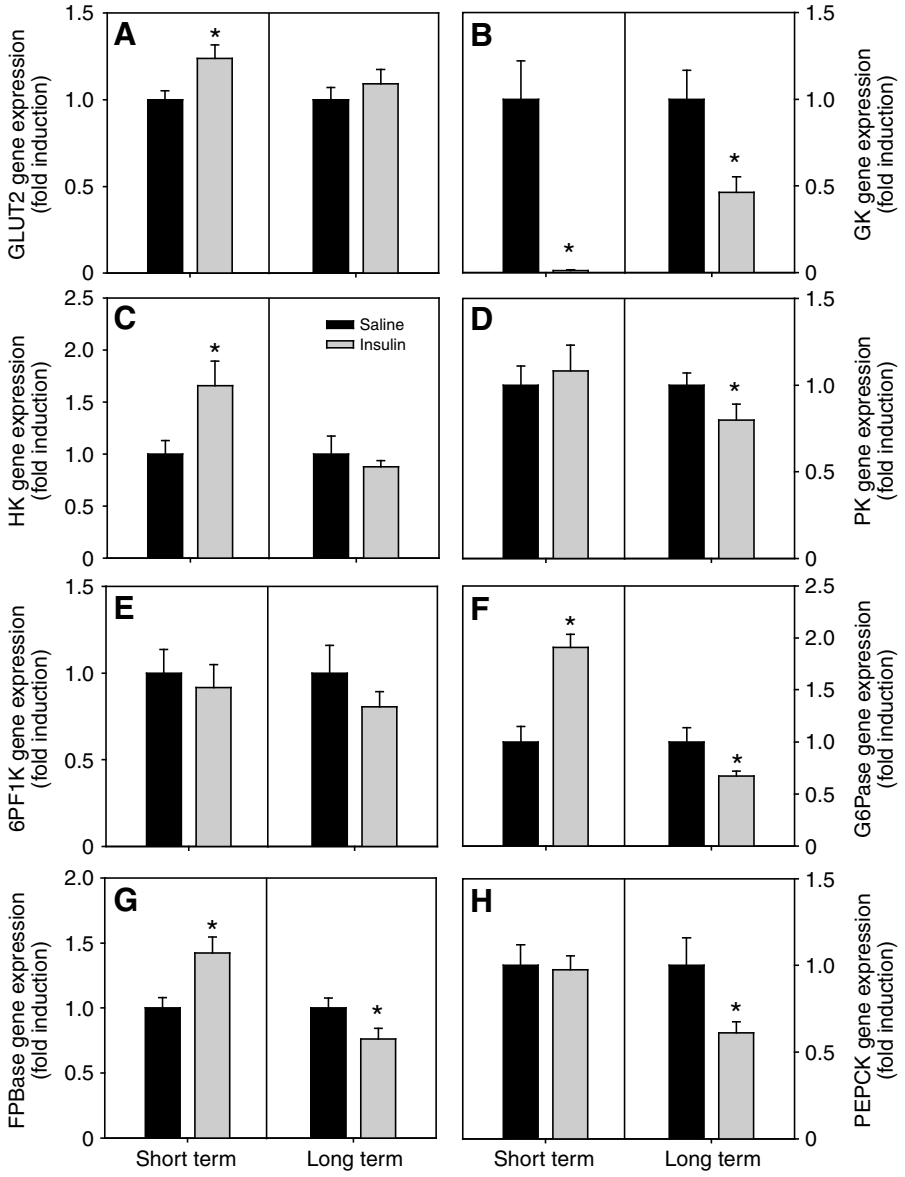


Fig. 4. Effects of intraperitoneal administration of insulin (6 h) or insulin implantation (4 days) on the level of mRNA transcripts encoding hepatic genes. (A) Glucose transporter type 2 (GLUT2), (B) glucokinase (GK), (C) hexokinase (HK), (D) pyruvate kinase (PK), (E) 6-phosphofructo-1-kinase (6PF1K), (F) glucose 6-phosphatase (G6Pase), (G) fructose 1,6-bisphosphatase (FBPase) and (H) phosphoenolpyruvate carboxykinase (PEPCK). mRNA levels were estimated using real-time RT-PCR. Expression values were normalized with elongation factor 1α (EF1α) transcripts and are indicated as fold variation of the saline solution-treated group. Results are expressed as means ± s.e.m. ($N$=8) and were analysed by one-way ANOVA followed by Student–Newman–Keuls comparison test. *Significant difference ($P$<0.05). For more details see Fig. 2.

largely responsible for the decreased systemic glucose uptake during hypoglycaemia, contributing in an important manner to counter-regulate it (Meyer et al., 2005). In agreement with this decrease in GLUT transcript levels, glycolytic enzymes like HK or PK were also inhibited.

Glycogen levels decreased in liver after insulin injection in the present study (despite increased Akt phosphorylation), probably as a result of the enhanced GPase activity and the absence of changes in GSase activity. Although hepatic glycogen levels after insulin administration in fish change in a highly variable manner (Mommsen and Plisetskaya, 1991), most studies using physiological insulin doses show decreased glycogen concentrations (Navarro et al., 2006). Thus, in fish as in mammals (Lecavalier et al., 1989), glycogen breakdown seems to be the primary mechanism responsible for the increase in endogenous glucose production during the initial recovery from insulin-induced hypoglycaemia. Other coordinated responses in trout hepatic metabolism were also observed in the short-term to deal with the insulin-induced hypoglycaemia. For example, we found that while GK transcript levels were strongly down-regulated, G6Pase transcripts were up-regulated in insulin-injected trout. Although in other fish (Egea et al., 2007) and mammals (Iynedjian, 2009) GK is always up-regulated by insulin, in the present study its repression could be related to the counter-regulation during hypoglycaemia, blocking the large glucose entrance and phosphorylation. In the same sense, the increased G6Pase transcript level probably improves glucose export to the bloodstream, as observed in the mammalian liver under hypoglycaemic conditions (Bady et al., 2002). As a whole, it seems that the glucose/glucose 6-phosphate cycle in fish liver is strictly regulated to meet the glucose requirements under insulin-induced hypoglycaemia, favouring glucose export and limiting glucose entry and phosphorylation to maintain normoglycaemia. These results are also supported by increased transcript levels of FBPase and GLUT2, the last probably favouring glucose export into the blood. The fact that PEPCK transcripts remain unaltered in insulin-injected fish may be related to a delayed increase in *de novo* glucose production, in contrast to the rapid glycogenolytic process (1–3 h) during the counter-regulation to hypoglycaemia (Caprio et al., 1992).

Globally, we found that when acute hypoglycaemia is produced by physiological insulin doses, rainbow trout are able to deal with

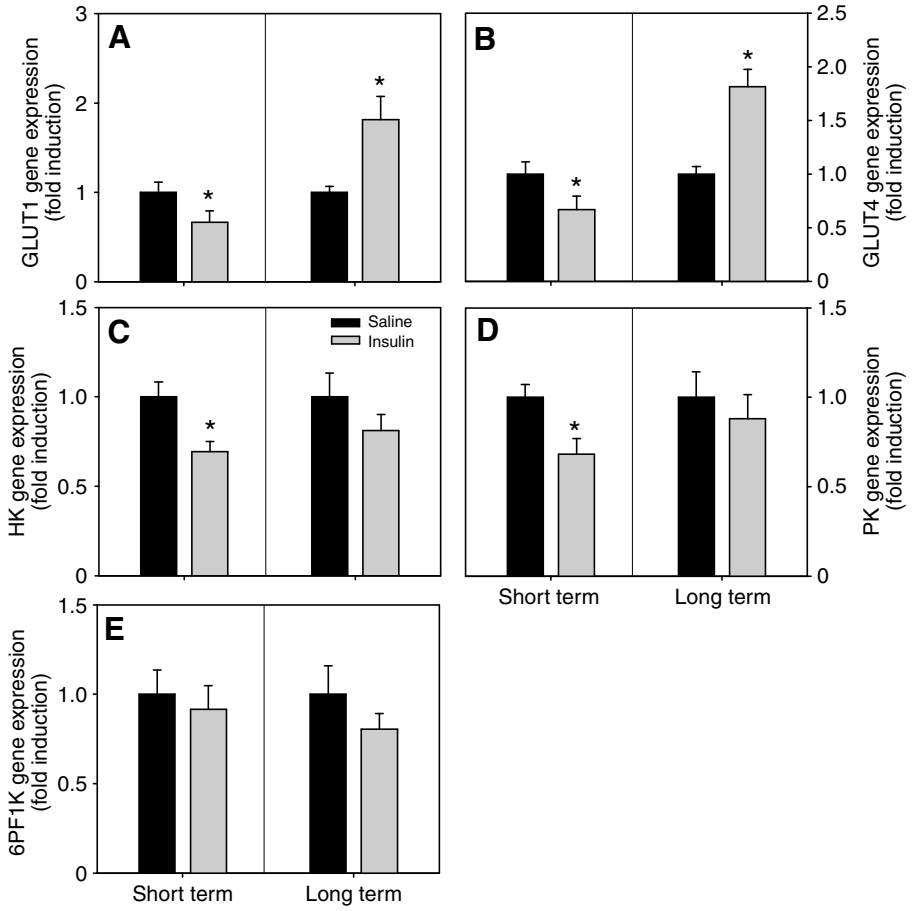


Fig. 5. Effects of intraperitoneal administration of insulin (6 h) or insulin implantation (4 days) on the level of mRNA transcripts encoding muscle genes. (A) glucose facilitative transporter type 1 (GLUT1), (B) glucose facilitative transporter type 4 (GLUT4), (C) HK, (D) PK and (E) 6PF1K. mRNA levels were estimated using real-time RT-PCR. Expression values were normalized with elongation factor 1α (EF1α) transcripts and are indicated as fold variation of the saline solution-treated group. Results are expressed as means ± s.e.m. ($N$=8) and were analysed by one-way ANOVA followed by Student–Newman–Keuls comparison test. *Significant difference ($P$<0.05). For more details see Fig. 2.

the fall in plasma glucose levels in a very efficient manner. This process includes a strong and rapid hepatic glycogenolytic response, but also molecular changes in major enzymes involved in glucose metabolism. In addition, and despite the large glycogen storage (probably taking place in the first hours of insulin exposure), decreased mRNA levels of GLUTs and glycolytic enzymes (probably reflecting depressed glucose transport and utilization) in the white muscle of trout were found, which could also be taking place in response to the hypoglycaemia. Thus even though the basis of this response in fish remains to be elucidated, the increase in the level of glycogenolytic hormones (adrenaline or glucagon) as a result of the insulin-induced hypoglycaemia (Matty and Lone, 1985) seems to be the more logical explanation. Our results show that under physiological insulin treatment most of the results obtained in other studies with pharmacological doses are not observed, probably due to a saturation of the system under high insulin levels (Ince, 1983a) which may mask the counter-regulatory natural response to hypoglycaemia by the trout.

### Chronic insulin administration: metabolic regulation in liver and muscle

The long-term regulation of glycaemia and the effects of chronic insulin infusion on metabolic-related genes was studied in trout implanted with osmotic mini-pumps that delivered bovine insulin at 1.4 IU $kg^{-1}$ (or 0.35 IU $kg^{-1}$ $day^{-1}$), corresponding to the same amount of hormone administered in a single injection for the acute trial.

As far as we are aware, this is the first study in fish in which Akt phosphorylation status has been assessed in the long-term in both skeletal muscle and liver. Surprisingly, we found no activation of Akt phosphorylation in muscle after this insulin infusion. Although insulin levels in the present study were low, Akt phosphorylation in the liver was activated, indicating that this level could also be sufficient to activate this protein in skeletal muscle. The absence of phosphorylation of Akt was also described in skeletal muscle of mammals during chronic insulin infusion, where it was considered a symptom of insulin resistance (Del Prato et al., 1994; Ueno et al., 2005). However, Ueno and colleagues showed that after 5 days of insulin infusion the rat liver also became insulin resistant, like the muscle (Ueno et al., 2005), while Del Prato and colleagues did not find any such symptoms during 3 days of hyperinsulinaemia (Del Prato et al., 1994). From these data, we can hypothesize that trout liver is not insulin resistant after 4 days of insulin stimulation, although the liver may become resistant if the hyperinsulinaemia is maintained for a longer period. Whereas in liver insulin stimulates Akt phosphorylation and inhibits *de novo* glucose production, in muscle Akt phosphorylation is not stimulated, but in contrast glucose transport and storage are enhanced. In mammals persistent hyperinsulinaemia leads to impaired insulin signalling through Akt, glucose transport, storage and glucose utilization in muscle (Bertacca et al., 2005; Del Prato et al., 1994). In this context, Akt phosphorylation in muscle seems to correlate with different biological effects on insulin-sensitive processes in trout in comparison with mammals.

Rainbow trout subjected to a continuous mild insulin infusion over 4 days exhibited hypoglycaemia as observed during the acute insulin treatment. However, this glycaemic profile may not result from the same metabolic events in the two cases. In contrast to the acute trial where the hypoglycaemia was linked primarily to an increased muscle glycogenesis, during the chronic insulin treatment

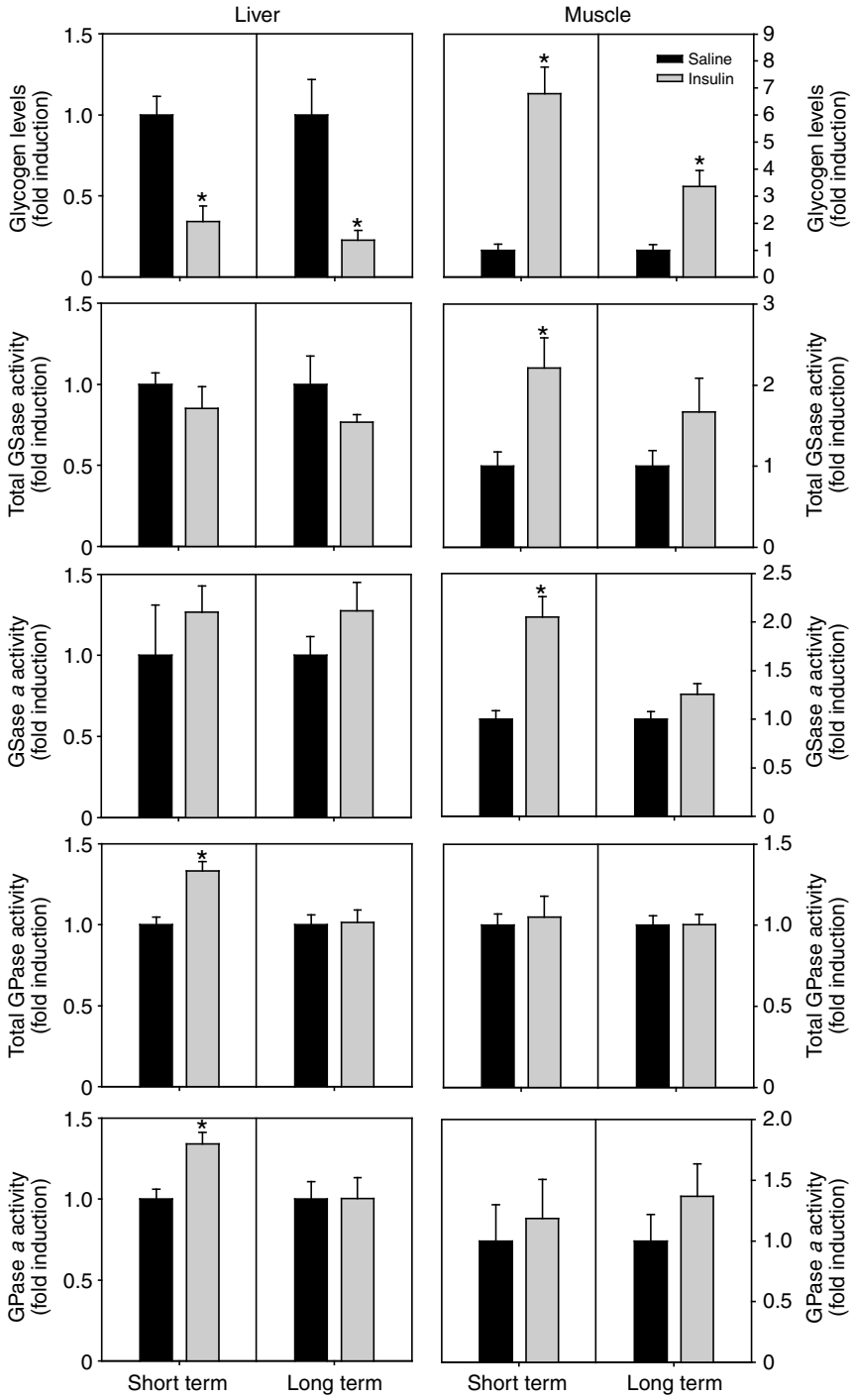


Fig. 6. Effects of intraperitoneal administration of insulin (6 h) or insulin implantation (4 days) on the liver and muscle glycogen levels, total glycogen synthase (GSase), activated glycogen synthase (GSase *a*), total glycogen phosphorylase (GPase), and activated glycogen phosphorylase (GPase *a*) of rainbow trout. GSase activity (mU mg$^{-1}$ protein) in controls was: 155.69±10.95 (liver i.p.), 132.95±20.84 (liver pump), 98.32±10.35 (muscle i.p.) and 45.55±52.63 (muscle pump). GPase activity (mU mg$^{-1}$ protein) in controls was: 768.80±34.92 (liver i.p.), 883.94±53.88 (liver pump), 1892.55±133.60 (muscle i.p.) and 1347.32±81.11 (muscle pump). Data are indicated as fold variation of the saline solution-treated group. Results are expressed as means ± s.e.m. ($N$=8) and were analysed by one-way ANOVA followed by Student–Newman–Keuls comparison test. *Significant difference ($P$<0.05). For more details see Fig. 2.

the hypoglycaemia is most probably a result of a depressed hepatic gluconeogenesis coupled to a persistent muscle glycogenesis. Moreover, this hypoglycaemia could be reinforced by an enhanced glucose uptake and storage in muscle (caused by a direct insulin action) which was not observed during the short-term treatment, probably due to the urgency of the counter-regulatory process against hypoglycaemia.

Despite the important differences in insulin levels in plasma in the acute (28.53 ng ml$^{-1}$) and the chronic (1.52 ng ml$^{-1}$) treatments, fish from the two groups displayed the same degree of hypoglycaemia (about 2 mmol l$^{-1}$). Unlike in the acute treatment where both hepatic and muscle metabolic reorganization were observed in order to deal with an acute hypoglycaemia, during the chronic insulin infusion the hepatic metabolism seems not be subject to the same counter-regulatory hyopglycaemic urgency. Although GK gene expression and glycogen levels are still down-regulated under chronic insulin stimulation, the degree of inhibition is less than that during the acute treatment. In fact, no changes were noticed in either GSase or GPase activity suggesting that the glycogen metabolism is close to equilibrium and glycogen stores are no longer potentially depleted. In agreement with this, we also found that G6Pase transcript levels were not enhanced to facilitate the export of glucose but in contrast were down-regulated by insulin. Moreover, the global down-regulation of gluconeogenic transcripts probably

indicates a decreased potential for *de novo* glucose production, as described in mammals (McCormick et al., 1979).

After 4 days of insulin infusion, as during the acute insulin treatment, we found that the muscle played a critical role in the events responsible for the hypoglycaemic effects of the hormone. Thus, enhanced glucose transport and storage were found during the chronic insulin infusion. This large glucose uptake and metabolism are probably responsible for an important part of the hypoglycaemic effect of insulin in fasted trout, since the muscle represents a large percentage of total fish mass and constitutes the main target for insulin (see 'Plasma glycaemia regulation by insulin in cannulated trout'). As mentioned above, despite the insulin resistance observed at the intracellular signalling level (degree of Akt phosphorylation), global muscle metabolism is clearly implicated in the uptake (increased GLUT gene expression) and storage of glucose (enhanced glycogen deposition). As a whole, the metabolic response observed in the trout white muscle is consistent with the classic anabolic role of insulin in mammals, including improved glucose uptake and storage, and the maintenance of oxidative glucose pathways (like glycolysis).

## Conclusions and perspectives

Taken together, the results obtained in the present study indicate that the fasted rainbow trout is well adapted to respond to both acute and chronic increases in circulating insulin levels, and that this hormone is able to improve glucose distribution and uptake by peripheral tissues, enhancing the capacity of the animal to deal with a glucose load. In addition, we found that in order to counter-regulate the insulin-induced hypoglycaemia following acute insulin administration, trout metabolism is strongly modified, making it difficult to meet some of the effects traditionally attributed to this hormone. However, the natural response to hypoglycaemia obtained in the present study allows us to validate our results in contrast to those in which insulin effects, while valid, were evaluated by employing pharmacological insulin doses, where saturation of the system may have been achieved. Despite the extremely low glucose intake of the trout under natural conditions, the present study demonstrates that this carnivorous fish species is able not only to maintain stable plasma glucose levels during long fasting periods but also to initiate short-term efficient responses to hypoglycaemia, composed of a rapid, coordinated response that includes skeletal muscle and liver metabolic reorganization. On the other hand and despite the insulin resistance observed in the muscle at the signalling level, some of the functions traditionally attributed to insulin were observed during the chronic insulin treatment, including increased glucose uptake and storage (mostly in the skeletal muscle) capacities as well as decreased *de novo* glucose production (in the liver). These results suggest that even when some evidence of counter-regulation to hypoglycaemia remains, the chronic exposure of rainbow trout to physiological insulin doses allows it to demonstrate several insulin effects that remain inaccessible under acute physiological or pharmacological doses. Finally, data obtained in the present study do not support the idea that the glucose intolerance exhibited by carnivorous fish like rainbow trout is related to a low insulin sensitivity or insulin resistance in the fasted state. In contrast, we demonstrate that this fish species presents most of the metabolic components to efficiently utilize glucose in the appropriate insulin context. In this sense, the fact that rainbow trout, even having higher insulin levels than mammals (Mommsen and Plisetskaya, 1991) and good insulin sensitivity in tissues over both the short and long term (this study), remains glucose intolerant represents for us a paradoxical situation. However, there are two observations that are worth mentioning. The first is the fact that current insulin radioimmunoassays in salmonids are possibly measuring proinsulin (the biological activity of which remains to be elucidated) (Plisetskaya, 1998), and then over-estimating real plasma insulin levels. In this context, it is possible that fish remain glucose intolerant just because they are partially insulin deficient. The second is that, although the use of fasted fish allows us to obtain a clearer response to exogenous insulin, we cannot ignore the fact that numerous factors interacting in the postprandial state are not present here. Then, even in the right insulin context, glucose intolerance in trout could still be influenced by other factors including the interaction with other hormones (i.e. somatostatins, glucagon) or nutrients (glucose, amino acids).

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 6PF1K-L | 6-phosphofructo-1-kinase, liver isoform |
| 6PF1K-M | 6-phosphofructo-1-kinase, muscle isoform |
| EF1α | elongation factor 1α |
| FBPase | fructose 1,6-bisphosphatase |
| G6Pase | glucose 6-phosphatase |
| GK | glucokinase |
| GLUT1 | glucose transporter type 1 |
| GLUT2 | glucose transporter type 2 |
| GLUT4 | glucose transporter type 4 |
| GPase | glycogen phosphorylase |
| GSase | glycogen synthetase |
| HK | hexokinase |
| PEPCK | phosphoenolpyruvate carboxykinase |
| PK-L | pyruvate kinase, liver isoform |
| PK-M | pyruvate kinase, muscle isoform |

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