The effects of alanine on glucose metabolism in rainbow trout: Integration of glucose fluxes and molecular evidence

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ABSTRACT

This thesis investigates the effects of alanine on rainbow trout's glucose metabolism at the organismal and molecular levels. Rainbow trout is an important aquaculture species that belongs to the salmonid family. As a carnivorous fish, the requirement of protein/amino acids in trout's diet is high. In contrast, rainbow trout are poor utilizers of carbohydrates. One prevalent hypothesis suggests that high levels of dietary amino acids could indeed contribute to the poor utilization of carbohydrates in this species. In mammals, there is evidence supporting the importance of alanine as a gluconeogenic precursor. However, a recent study found that alanine stimulates hepatic AMP-activated protein kinase (AMPK) to lower circulating glucose levels in mice. Alanine levels are high in all tissues in rainbow trout. The role of alanine in gluconeogenesis is less clear in trout and there is no evidence, to our knowledge, regarding its effects on glucose kinetics. Therefore, the main goal of the study was to investigate the impact of the continuous infusion of exogenous alanine for 4h on glucose fluxes and to identify potential mechanisms in tissues that could interpret the observed changes in glucose fluxes *in vivo*. Glucose turnover, appearance and disposal, R_t, R_a and R_d, respectively, were measured to determine the impact of alanine on glucose fluxes. The expression and/or activity of key genes in glucose transport, utilization and gluconeogenesis were assessed in liver and muscle. An additional goal was to assess whether alanine activates AMPK in trout. The levels of phosphorylated AMPK and other signaling proteins known to interact with the latter were quantified. Results show that alanine reduced plasma glucose levels and inhibited R_a and R_d glucose, consistent with previously observed effects of insulin in rainbow trout. The

reduction in the expression of a paralogue of *glut4*, a key gene in glucose transport, and the activity of hexokinase (HK), a key enzyme in glucose utilization, in muscle can partially explain the observed reduction in R_d glucose. Together, these results suggest that glucose was not a preferred substrate under conditions of increased alanine availability and that alanine was probably oxidized to provide energy. Alanine failed to activate AMPK in trout, contrary to mammalian findings. However, it increased AKT (also known as protein kinase B) phosphorylation in muscle, similar to the effect of insulin in trout. In conclusion, my results suggest that alanine mediated at least some of the observed effects by stimulating insulin secretion given the similarities between the effects of exogenous alanine and insulin in rainbow trout as discussed above. Future studies are warranted to investigate the hypothesis that alanine is an insulin secretagogue in rainbow trout.

RÉSUMÉ

Cette thèse étudie les effets de l'alanine sur le métabolisme du glucose chez la truite arc-en-ciel au niveau de l'organisme entier et moléculaire. La truite arc-en-ciel est une importante espèce d'aquaculture qui appartient à la famille des salmonidés. En tant que poisson carnivore, les besoins en protéines/acides aminés dans le régime alimentaire de la truite sont élevés. En contraste, la truite est une mauvaise utilisatrice de carbohydrates. Une hypothèse répandue suggère que des niveaux élevés d'acides aminés alimentaires pourraient en effet contribuer à la mauvaise utilisation des glucides chez cette espèce. Chez les mammifères, il y a des preuves qui supportent l'importance de l'alanine en tant que précurseur gluconéogénique. Cependant, une étude récente a montré que l'alanine stimule la protéine kinase activée par l'AMP (AMPK) dans le foie pour réduire les niveaux de glucose en circulation chez les souris. Les niveaux d'alanine sont élevés dans tous les tissus de la truite arc-en-ciel. Le rôle de l'alanine dans la gluconéogenèse est moins clair chez la truite et il n'y a pas de preuve, à notre connaissance, concernant ses effets sur la cinétique du glucose. Par conséquent, l'objectif principal de l'étude était de déterminer l'impact de l'infusion continue d'alanine exogène pendant 4h sur les flux de glucose et d'identifier les mécanismes potentiels dans les tissus qui pourraient interpréter les changements observés dans les flux de glucose. Le taux de renouvellement, l'apparence et l'utilisation du glucose, R_t, R_a et R_d, respectivement, ont été mesurées pour déterminer l'impact de l'alanine sur les flux de glucose. L'expression et/ou l'activité de gènes clés dans le transport, l'utilisation et la gluconéogenèse du glucose ont été évaluées dans le foie et le muscle. Un autre objectif était d'évaluer si l'alanine active l'AMPK chez la truite. Les niveaux d'AMPK phosphorylé

et d'autres protéines de signalisation connues d’interagir avec cette dernière ont été quantifiés. Les résultats montrent que l'alanine a réduit les niveaux de glucose dans le plasma et a inhibé le glucose R_a et R_d, ce qui correspond aux effets de l'insuline observés précédemment chez la truite. La réduction de l'expression d’un paralogue de *glut4*, un gène clé dans le transport du glucose, et l'activité de l'hexokinase (HK), un enzyme clé dans l'utilisation du glucose, dans le muscle peuvent expliquer en partie la réduction observée du glucose R_d. Ensemble, ces résultats suggèrent que le glucose n'était pas un substrat privilégié dans des conditions de disponibilité élevée d'alanine et que ce dernier était probablement oxydé pour fournir de l'énergie. L'alanine n'a pas réussi à activer l'AMPK chez la truite, contrairement aux résultats obtenus chez les mammifères. Cependant, elle a augmenté la phosphorylation de l'AKT (également connue sous le nom de protéine kinase B) dans le muscle, similaire à l'effet de l'insuline chez la truite. En conclusion, mes résultats suggèrent que l'alanine a médié au moins certains des effets observés en stimulant la sécrétion d'insuline étant donné les similarités entre les effets de l'alanine exogène et de l'insuline chez la truite, comme indiqué plus haut. Des études futures sont nécessaires pour étudier l'hypothèse selon laquelle l'alanine est un sécrétagogue de l'insuline chez la truite arc-en-ciel.

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Chapter 1: General Introduction

Introduction

This thesis investigates the effects of exogenous alanine on glucose fluxes and key molecular indices of glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). The carnivorous diet of these salmonid fish is high in proteins/amino acids and lipids, but comparatively low in carbohydrates. Regrettably, little is known about the effects of individual amino acids on glucose metabolism in any species. Only a few studies have investigated the effects of alanine on glucose metabolism *in vitro* (Canals et al., 1992; Pereira et al., 1995) or after a dietary treatment (Cowey et al., 1977b; Kirchner et al., 2003a). A recent mammalian study found that an oral gavage with alanine stimulated AMP-activated protein kinase (AMPK), mainly in liver, and lowered circulating glucose levels *in vivo* (Adachi et al., 2018). Therefore, the general aim of this thesis is to measure the impact of alanine on glucose metabolism in rainbow trout, a carnivorous species considered to be a poor utilizer of carbohydrates, and to investigate whether the potential effects of this amino acid are also linked to AMPK activation in this species.

Metabolic fuels

Adenosine triphosphate (ATP) is the direct energy source used by living organisms to sustain cellular work, but it is only present in small amounts because cells cannot store it in large quantities (Weber, 2001). Therefore, metabolic fuel reserves (lipids, carbohydrates and to a lesser extent proteins) are constantly mobilized to generate ATP and match its rate of utilization for cellular needs (Weber, 2001). Each metabolic fuel has advantages and disadvantages that animals exploit or minimize, respectively, by strategically selecting various fuel mixtures (Weber, 2011). For instance, lipids are ideal for low- to moderate-intensity tasks because they are stored

dehydrated, in large quantities, and yield the most energy per gram (Weber, 2011). However, lipids are not soluble in aqueous fluids and depend on transport proteins in the plasma and cytosol. By contrast, carbohydrates are suitable for short-term intense tasks because their maximal rate of ATP production is high, they are soluble in water, and can also be used under anaerobic conditions (Weber, 2011). Yet, carbohydrates are not stored in large quantities and can consequently only be used for a limited duration. Most cellular proteins are not used as oxidative fuels because they play important roles in force generation, structural support, regulation (enzymes and ion pumps) and their catabolism generate toxic ammonia (Weber, 2001; Weber, 2011). Carnivorous animals must rely more on proteins for ATP production than those eating other diets. Under extreme conditions such as extended starvation or in the late stages of long migrations in salmon, proteins can become the exclusive oxidative fuel (Weber, 2001).

Glucose as a metabolic fuel

Glucose is an important fuel for mammalian tissues. For instance, the brain mainly relies on glucose as a metabolic fuel rather than on alternative substrates that either have a much lower concentration (ketone bodies) or whose transport is limited across the blood-brain barrier (free fatty acids) (Alsahli et al., 2017). Blood glucose levels are monitored and maintained stable through multiple mechanisms in mammals (Polakof et al., 2011c). Even though glucose is key in mammalian energy metabolism, its importance to fish is less clearly characterized (Hemre et al., 2002). Generally, circulating glucose levels, turnover and oxidation rate, are lower in fish compared to mammals and birds, except possibly for American eel and skipjack tuna (Hemre et al.,

2002; Polakof et al., 2011c; Polakof et al., 2012). The lower glucose utilization of fish was suggested to be related to ectothermy (Polakof et al., 2012). Teleost fishes are a diverse group that consists of more than 20,000 species (Polakof et al., 2012) and their capacity to use glucose also seems to vary in a species-specific manner. Salmonids are important economically (aquaculture) and among the best studied teleost fishes, and carnivorous species like rainbow trout are known for their poor capacity to use glucose. Therefore, their diet is low in carbohydrates and unusually high amounts (>20% of the diet) have been associated with decreased growth as a result of persistent postprandial hyperglycemia (Polakof et al., 2012). Nevertheless, significant changes in circulating glucose levels caused by different stresses suggest that glucose may still be important for trout (Polakof et al., 2012). This notion is further supported by the fact that glucose utilization per gram of tissue was found to be the highest in brain followed by gonads and red blood cells in rainbow trout (Washburn et al., 1992). After glucose uptake inside the cell through facilitative glucose transporters (GLUTs), this molecule can be utilized for ATP production through the glycolytic pathway, converted to lipids or stored as glycogen (Enes et al., 2009). In fish as in all organisms, glucose is phosphorylated by hexokinase (HK), known as glucokinase (GK) in liver, which catalyzes the first step in glycolysis (see Fig. 1.1 for a schematic representation of the pathway). Under aerobic conditions, the last step in this pathway is catalyzed by pyruvate kinase (PK) (Enes et al., 2009).

Sources of de novo glucose production

After eating and during short-term fasting, the liver produces glucose mainly through glycogen breakdown (glycogenolysis). When fasting is more prolonged and

glycogen reserves become depleted, hepatic glucose production progressively relies more on gluconeogenesis (Bechmann et al., 2012; Chung et al., 2015; Zhang et al., 2019). As in mammals, therefore, glycogenolysis and gluconeogenesis are responsible for *de novo* glucose production in fish (Enes et al., 2009). Natural food deprivation caused by seasonal fluctuations in food supply, aestivation or anorexic spawning migration are common in most fish (Hemre et al., 2002). Glycogen breakdown is catalyzed by glycogen phosphorylase (GP) in fish liver (Enes et al., 2009). Gluconeogenesis synthesizes new glucose molecules from other substrates including gluconeogenic amino acids, lactate and glycerol (Polakof et al., 2012; Zhang et al., 2019), that can eventually be converted to glycogen (Moon, 1988). The key gluconeogenic enzymes are phosphoenolpyruvate carboxykinase (PCK), fructose-1,6-biphosphatase (FBP) and glucose-6-phosphatase (G6Pc) in mammals (Bechmann et al., 2012; Chung et al., 2015; Zhang et al., 2019) and fish (Enes et al., 2009). G6P catalyzes the last step, conversion of glucose-6-phosphate to glucose, of both glycogenolysis and gluconeogenesis (see Fig. 1.1 for a schematic representation of the pathway) (Enes et al., 2009). Many factors affect endogenous glucose production including substrates availability, hormonal modulation, and the activity of regulatory enzymes (Chung et al., 2015; Enes et al., 2009; Kirchner et al., 2008; Zhang et al., 2019). Differently from omnivorous mammals, the expression and activity of the key gluconeogenic enzymes are not regulated by carbohydrates in rainbow trout (Kirchner et al., 2008; Marandel et al., 2015). Fasting and dietary protein were suggested to better regulate gluconeogenic enzymes in trout (Enes et al., 2009; Kirchner et al., 2008) and carnivorous mammals, such as cats for instance (Schermerhorn, 2013).

In mammals, the liver, kidney, and small intestine mainly contribute to glucose release in the circulation (Mithieux et al., 2006). Liver is the main gluconeogenic organ (Chung et al., 2015), but the supply from kidney and small intestine was found to increase with fasting and in diabetes (Mithieux et al., 2006). Additionally, the small intestine was suggested to indirectly promote glucose appearance by supplying gluconeogenic substrates (lactate and alanine) to the liver (Mithieux et al., 2006). In rainbow trout, the liver and to a lower degree the kidney, were found to be the main sites of gluconeogenesis (Cowey et al., 1977b; Knox et al., 1980). The kidney was estimated to contribute for approximately 10% of total glucose production at rest in hepatectomized rainbow trout indicating that the liver is the main site of glucose production (Haman et al., 1997a). Activation of alanine aminotransferase (ALT) plays an important role in increasing gluconeogenesis from amino acids, particularly from alanine (Sánchez-Muros et al., 1998). Under starvation and when feeding on a high protein diet, ALT activity increased in rainbow trout liver but not in kidney (Sánchez-Muros et al., 1998). In addition to the liver and kidney, key gluconeogenic enzymes were also found in intestine in rainbow trout (Kirchner et al., 2005; Kirchner et al., 2008).

Glucose kinetics theory

Circulatory metabolites undergo constant turnover (production and utilization) (Weber and Zwingelstein, 1995), and changes in the concentration of plasma metabolites has been commonly used to draw conclusions about fluxes. Changes in concentration indicate an imbalance between the release and uptake of a given metabolite (Weber and Zwingelstein, 1995). However, flux and concentration do not change in a similar way because big variations in release and uptake can lead to an

increase, no change or a decrease in concentration depending on the difference between the rate of appearance (R_a) and disposal (R_d) (Haman and Weber, 1996). Therefore, metabolite fluxes are more informative than plasma concentration.

The continuous tracer infusion technique was developed for use in rainbow trout and its reliability to measure glucose fluxes was validated in this species (Haman and Weber, 1996; Haman et al., 1997a). The steady and non-steady state equations of Steele (Steele, 1959) were used to calculate glucose fluxes. During continuous infusion, a priming dose of labeled glucose is administered about 60 min before starting an experiment because otherwise, it would take several hours to reach isotopic steady state given that the glucose pool of vertebrates is large (Haman and Weber, 1996). Isotopic steady state is reached when the ratio of labeled to unlabeled glucose (or the specific activity) remains constant. Glucose pool can be defined as being mostly the glucose dissolved in extracellular fluids (plasma and interstitial fluids) (Steele et al., 1956). The 60 min period is needed to make sure that isotopic steady state has been reached because mixing is not instantaneous (Steele, 1959; Steele et al., 1956). In rainbow trout, isotopic steady state is usually reached in <45 min (Choi and Weber, 2015). The Steele equations used to calculate R_a and R_d were derived to explain how glucose behaves in a two-compartment model: the rapidly mixing and the slowly mixing glucose pool compartments (Steele, 1959). The volume of glucose in the plasma and extracellular tissue fluids mixes rapidly. The intracellular volume of tissues represents the slowly mixing compartment. After the priming dose is well mixed within the glucose pool, the rate at which labeled glucose is infused equals the rate at which it leaves the rapidly mixing pool and therefore a steady state is reached. When the infusion rate of

labeled glucose (F in Bq kg^{-1} min^{-1}) and the specific activity (SA in Bq μmol^{-1}) are known, the turnover rate (R_t in µmol kg^{-1} min^{-1}) can be calculated using the steady state equation of Steele,

$$Rt = Ra = Rd = \frac{F}{SA}$$

A change in the concentration occurs when there is a mismatch between R_a and R_d. Under these conditions, R_a and R_d can be calculated individually using the non-steady state equations of Steele,

$$Ra = \frac{F - pV\left(\frac{C2 + C1}{2}\right)\left(\frac{SA2 - SA1}{t2 - t1}\right)}{\left(\frac{SA2 + SA1}{2}\right)}$$

$$Rd = Ra - pV\left(\frac{C2 - C1}{t2 - t1}\right)$$

In these non-steady state equations, pV is the glucose pool volume (ml kg^{-1}), C1 and C2 represent the plasma glucose concentration (mmol l^{-1}) at times t1 and t2 (in min). A glucose pool volume of 50 ml kg^{-1} was used for calculating the non-steady state fluxes as previously validated in trout (Haman et al., 1997b).

Amino acids

The main function of amino acids is the synthesis of proteins and nonprotein nitrogenous compounds (Blanco and Blanco, 2017). Thus, the catabolism of amino acids for ATP generation is a secondary role that increases during fasting or with high dietary protein intake given that proteins are not stored unlike carbohydrates and lipids

(Blanco and Blanco, 2017; Ruderman, 1975). Dietary proteins/amino acids promote insulin secretion and therefore increase glucose uptake by peripheral tissues (Rietman et al., 2014). However, prolonged intake of high dietary proteins, especially those high in branched-chain amino acid (BCAA) leucine, isoleucine and valine, is linked with increased risk of insulin resistance and type-2-diabetes (Rietman et al., 2014). High levels of plasma amino acids, especially BCAA, are linked to these diseases because they have a role in decreasing peripheral insulin sensitivity by causing hyperinsulinemia over time. Amino acids, and especially the BCAA leucine, activate the target of rapamycin complex 1 (TORC1) (Rietman et al., 2014). The latter is a seine-threonine protein kinase that is sensitive to nutrients and insulin signaling. TORC1 has important roles in many cellular processes such as protein synthesis, lipid synthesis, and energy storage. (Rietman et al., 2014). The link between BCAA and the risk for insulin resistance/diabetes can be partially explained via TORC1. This is because the overactivation of the TOR pathway can lead to the phosphorylation of insulin receptor substrate 1 (IRS1) via a negative feedback loop which decreases insulin sensitivity (Rietman et al., 2014).

Compared to a terrestrial carnivore such as the kitten, juvenile rainbow trout have a higher requirement for essential amino acids when these are expressed per unit of digestible energy (Panserat et al., 2013). Dietary protein requirement (% of the diet) of rainbow trout is high compared to other animals (see Table 1.1). In carnivorous animals like cat and rainbow trout, changes in dietary protein levels have minor effects on the activity of enzymes involved in amino acid catabolism (Kaushik and Seiliez, 2010). This lack of control is considered as a major reason for high protein requirement given that

amino acid catabolism generates nitrogen waste and reduces the amount of amino acids that can be devoted to protein synthesis (Panserat et al., 2013). A general hypothesis to explain the limited metabolic use of carbohydrates in rainbow trout postulates that the high levels of postprandial amino acids, which are in excess in trout's diet compared to the limited amount of carbohydrates, could negatively impact insulin action and glucose metabolism (Kirchner et al., 2003a; Kirchner et al., 2003b; Kirchner et al., 2005; Panserat et al., 2013). After being absorbed by the intestine, amino acids enter the portal blood stream and they can be used for protein synthesis, oxidation or they can be converted to glucose or fat (Walton and Cowey, 1982). The main roles of amino acids are described in the next section.

Roles of amino acids

Growth

The improvement of dietary protein/amino acid utilization and the increase of muscle protein growth is crucial in aquaculture species such as rainbow trout (Kaushik and Seiliez, 2010). The relative protein concentration for maximal growth rate is higher in the diet of fish as compared to terrestrial animals, but there is no or little difference in the absolute (expressed as unit metabolic mass per day) protein requirements (Kaushik and Seiliez, 2010). This has been traditionally explained by the lower energy requirements of ectothermic fish. In fact, maintenance energy requirements of trout are 10-20 times lower than endothermic terrestrial vertebrates (Panserat et al., 2013). Growth is promoted by protein synthesis, an anabolic process that uses amino acids as building units. The rate of protein synthesis varies between tissues with the highest rate in liver and the lowest in white muscle (Lall and Anderson, 2005). Compared to

mammals, white muscle protein synthesis is lower in fish (Lall and Anderson, 2005). However, most of the synthesized proteins are retained in white muscle in fish. Relative to other tissues, the rate of protein synthesis in rainbow trout muscle represents about 22%; however, muscle protein content accounts for about 50% of the body protein (Kaushik and Seiliez, 2010). TORC1 is an evolutionary conserved serine/threonine protein kinase that is involved in cell growth, which is fundamental for organismal growth, as well as other processes (Cornu et al., 2013; Kim and Kim, 2016). TORC1 regulates cell growth by the stimulation of protein synthesis via its effects on downstream effectors including p70 ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), which are involved in ribosome biogenesis and translation, respectively (Kim and Kim, 2016). TORC1 is sensitive to nutrients, mainly amino acids, insulin/insulin-like growth factor 1 and cellular energy (Cornu et al., 2013; Kim and Kim, 2016). One important amino acid involved in the activation of TORC1 is leucine (Kim and Kim, 2016). For instance, leucine increased insulin sensitivity and body weight by enhancing the phosphorylation of the insulin-stimulated protein kinase B (AKT) and TOR in different tissues, including the muscle, in rats fed a high fat diet (Li et al., 2013). In rainbow trout, TOR signaling has been studied *in vitro* and *in vivo* under different conditions (Dai et al., 2013; Dai et al., 2014; Dai et al., 2015; Lansard et al., 2010; Lansard et al., 2011). Feeding with trout commercial diet (high in protein) increased the phosphorylation of TOR and its main targets suggesting that TOR is conserved in fish (Seiliez et al., 2008). *In vitro*, insulin and amino acids were found to regulate TOR signaling (Seiliez et al., 2008). The activation of TOR and its main effectors were impaired by decreasing protein/carbohydrate ratio (Seiliez et al.,

2011). Leucine was found to activate TORC1 target, ribosomal protein S6 (S6), in the presence of insulin in trout hepatocytes suggesting that leucine might have a role in the activation of TOR pathway in fish similar to mammals (Lansard et al., 2011).

Metabolism

In mammals, amino acids are mainly used as building units for the synthesis of proteins (for structural support) and many nitrogenous compounds (including hormones, coenzymes, creatine, purines and pyrimidines) (Blanco and Blanco, 2017). Given the feeding habit of carnivorous fish like rainbow trout, amino acids represent an important energy source that can provide ATP for many physiological processes. This is possible for trout because the end product of nitrogen metabolism in fish is mainly ammonia which is less energetically demanding than converting it to urea or uric acid (Ballantyne, 2001). Moreover, the synthesis of storage molecules, like carbohydrates and lipids, for later use is more energetically requiring than the direct use of amino acids from the diet (Ballantyne, 2001). It was estimated that trout could transport six times the amount of amino acids required for protein synthesis indicating the capacity of these fish to supply amino acids not only for protein synthesis but also for other physiological processes like oxidation (Robinson et al., 2011). Amino acid catabolism is higher in fish, especially teleost, than mammals (Ballantyne, 2001). In the latter, the use of amino acids as a fuel is a secondary role that can be replaced by carbohydrates or fats (Blanco and Blanco, 2017).

Amino acid catabolism occurs in the liver, except for branched-chain amino acids (leucine, isoleucine and valine) that are mainly oxidized in the muscle in mammals and fish (Ballantyne, 2001; Blanco and Blanco, 2017). To initiate the catabolism of amino

acids, transamination (transfer) and deamination (separation) reactions must occur. Transamination is the transfer of the amine group from an amino acid to a keto acid (such as pyruvate, oxaloacetate or α-ketoglutarate) forming a new amino acid and a new keto acid (Blanco and Blanco, 2017). Transaminases or aminotransferases catalyze transamination, which is a reversible reaction. In salmonids, aspartate and alanine aminotransferases are quantitatively the most important, similar to other animals (Walton and Cowey, 1982). For instance, alanine aminotransferase catalyzes the transfer of the amine group from alanine to α-ketoglutarate to form pyruvate and glutamate using pyridoxal phosphate as a coenzyme (Blanco and Blanco, 2017). Pyruvate can be converted to glucose (or glycogen) through gluconeogenesis or to acetyl-CoA. The latter can either be oxidized to CO_2 and H_2O in the tricarboxylic acid (TCA) cycle or used for the synthesis of other substrates, such as fatty acids (Blanco and Blanco, 2017). Oxidative deamination liberates the amine group from an amino acid. Glutamate dehydrogenase removes the amine group from glutamate in the presence of coenzymes to generate ammonia (NH_3) and α-ketoglutarate (see Fig. 1.2 for a visual representation of these reactions) (Blanco and Blanco, 2017). These reactions have been similarly described in fish (Mommsen et al., 1980; Walton and Cowey, 1982).

Insulin secretion

In mammals, increases in plasma glucose levels trigger insulin secretion from pancreatic β-cells to promote glucose uptake, utilization and storage in many tissues and inhibit glucose production from the liver (Saltiel and Kahn, 2001). However, amino acids, as well as other nutrients, also stimulate β-cells and lead to the release of insulin

(Mann and Bellin, 2016). Some amino acids have better insulinotropic effect than others. In mammals, the main amino acids that have a role in insulin secretion are alanine, glutamine, glutamate, leucine and arginine (Newsholme et al., 2005). In many fish species however, amino acids are known to be better insulin secretagogue than glucose (Navarro et al., 2002; Panserat et al., 2013). In the teleost fish barfin flounder (*Verasper moseri*), arginine, alanine, methionine and serine produced higher plasma insulin levels, measured by a validated time-resolved fluoroimmunoassay, compared to glucose (Andoh, 2007). In coho salmon (*Oncorhynchus kisutch*), arginine, alanine and lysine elevated plasma insulin levels, measured by a homologous radioimmunoassay, and histidine decreased it when injected intraperitoneally (Plisetskaya et al., 1991). Moreover, an increase in plasma insulin levels were observed in rainbow trout, and two other salmon species, fed a diet supplemented with arginine (Plisetskaya et al., 1991). When insulin was co-injected with amino acids (threonine or alanine) or glucose, it reduced circulating levels of amino acids and glucose in barfin flounder (Andoh, 2007). These studies indicate that amino acids can mediate the release of insulin which promotes their disposal.

The role of alanine in metabolism

Alanine is considered as a key gluconeogenic amino acid in mammals (Felig, 1973) and fish (Jürss and Bastrop, 1995). Quantitatively, alanine is important in both species, especially in the postabsorptive state, after prolonged starvation or following exercise in man (Felig, 1973; Felig et al., 1970) and during spawning migration in sockeye salmon (Mommsen et al., 1980) or after exhaustive exercise in rainbow trout (Milligan, 1997). Felig and colleagues proposed the glucose-alanine cycle to explain the

relative predominance of alanine even when a general decrease in amino acid release in the circulation occurs after prolonged starvation in man (Felig, 1973; Felig et al., 1970). In this cycle, alanine was suggested to be synthesized in the muscle by the transamination of pyruvate (derived from glucose or metabolism of other amino acids) and released in the circulation to be taken up by the liver where it can be reconverted to glucose and urea. The researchers suggest that alanine contributes to gluconeogenesis if synthesized from pyruvate derived from non-carbohydrate precursors (Felig et al., 1970). In fish, it was similarly suggested that most of the amino acids are converted to alanine which is released from the white muscle and transported to other tissues, including the liver (Milligan, 1997; Mommsen et al., 1980). In fact, alanine was found to be the dominant amino acid in all tissues, except the brain, and it accounted for >50% of the total amino acid pool size in rainbow trout (Storey, 1991). In mammals, alanine, a neutral amino acid, can be taken up by cells through different transport systems, including via active transporters that are Na^+ dependent or facilitated diffusion transporters that are Na^+ independent (Blanco and Blanco, 2017). Alanine uptake in trout hepatocytes was found to be mostly mediated through the ASC system, one of the active transporters with Alanine, Serine and Cysteine as preferred substrates (Canals et al., 1992).

Adenosine monophosphate-activated protein kinase (AMPK)

AMPK is an evolutionary conserved protein kinase that functions as a cellular energy sensor (Jeon, 2016; Kjøbsted et al., 2018; Mihaylova and Shaw, 2011; Shaw, 2009). It is activated in situations of energy stress such as low nutrient availability, prolonged exercise and hypoxia that lead to an increase in AMP or ADP and a decrease

in ATP levels (Mihaylova and Shaw, 2011; Shaw, 2009). Additionally, it was described that AMPK can sense glucose levels by a non-canonical mechanism (not dependent upon changes in adenine nucleotide levels) that leads to the activation of AMPK when glucose levels are depleted (Hardie, 2018). At the metabolic level, AMPK stimulates catabolic pathways that produce ATP and inhibits the ATP consuming anabolic pathways involved in the synthesis and storage of macromolecules in order to restore energy homoeostasis (Hardie, 2018). To re-establish ATP levels, AMPK enhances many cellular processes including glucose uptake, glycolysis, glycogen breakdown (by activating glycogen phosphorylase), fatty acid uptake and oxidation (Hardie, 2018; Jeon, 2016). On the other hand, AMPK inhibits many targets including proteins involved in lipid synthesis, gluconeogenesis, glycogen synthesis (through inactivation of glycogen synthase), and protein synthesis (through the inhibition of TORC1) (Hardie, 2018; Jeon, 2016). AMPK target proteins are activated or inhibited by phosphorylation. This protein kinase phosphorylates its targets by transferring a phosphate group of an ATP molecule on the aliphatic side chain of an amino acid with a hydroxyl group (like serine and threonine) (Hardie, 2018). In a piscine model like rainbow trout, studies suggest an evolutionary conserved role of AMPK in the regulation of energy homeostasis. Changes at the gene expression, protein and enzyme activity levels were found under different physiological conditions (fasting and re-feeding cycle and exercise) or pharmacological manipulations (AICAR and metformin) in liver (Polakof et al., 2011a) and skeletal muscle (Magnoni et al., 2012; Magnoni et al., 2014; Morash et al., 2014).

AMPK signaling pathway

AMPK is a heterotrimeric complex containing a catalytic α subunit and two regulatory subunits, β and γ (Hardie, 2018; Jeon, 2016; Kjøbsted et al., 2018; Mihaylova and Shaw, 2011; Shaw, 2009). The catalytic α subunit carries the activation loop at threonine 172 (T172). The β subunit provides structural support for the binding of the α and γ subunits and it comprises a carbohydrate-binding module that senses and binds to glycogen. The γ subunit contains binding sites for adenine nucleotides like AMP, ADP and ATP. Liver kinase B1 (LKB1) is the main upstream kinase catalyzing AMPK phosphorylation on T172 (Hardie, 2018; Kjøbsted et al., 2018; Shaw, 2009). Calcium/calmodulin-dependent protein kinase kinase 2 ($CaMKK_2$) also mediated AMPK activation when intracellular Ca^{2+} levels increase (Hardie, 2018; Jeon, 2016). Other protein kinases and phosphatases regulate AMPK (Jeon, 2016; Kjøbsted et al., 2018; Mihaylova and Shaw, 2011). AMPK detects changes in AMP/ATP (primarily) and ADP/ATP ratios. AMP binding within the γ subunit induces a conformational change (more compact) leading to AMPK activation by promoting its phosphorylation at T172 by LKB1, protecting it from being dephosphorylated at T172 by phosphatases and increasing its activity (Hardie, 2018). The most active form of AMPK prevails when it is phosphorylated at T172 in the α subunit and bound to AMP at both sites in the γ subunit (Craig et al., 2018). Under nutrient-rich conditions, AMPK is inhibited by phosphorylation at specific sites by several proteins. Here, relevant proteins, measured for the purpose of this thesis, will now be discussed below. Specifically, AMPK can be repressed by direct phosphorylation mediated by protein kinase B (AKT) after increases in insulin levels (Jeon, 2016; Kjøbsted et al., 2018). AKT is a key protein kinase in the insulin

signaling cascade that mediates many of insulin functions (Taniguchi et al., 2006). AKT phosphorylates AMPK α subunit on specific serine residues (species and isoform dependent) to prevent kinases from phosphorylating T172 and thus it hinders AMPK activation (Craig et al., 2018; Jeon, 2016; Kjøbsted et al., 2018). TORC1 is another protein kinase that interacts with AMPK and has opposing roles compared to the latter (Hardie, 2018; Shaw, 2009). Contrary to AMPK, TORC1 is activated under nutrient-replete conditions, especially by amino acids, to stimulate several processes including the synthesis of proteins involved in cell growth, an energy requiring pathway, via the phosphorylation of its targets (Hardie, 2018; Kjøbsted et al., 2018). AMPK inhibits TORC1 by different mechanisms (Hardie, 2018; Kjøbsted et al., 2018). On the other hand, AKT activates TORC1 (Hay, 2011). In summary, when ATP is available, AKT and TORC1 are activated and AMPK is inhibited. For a visual representation of AMPK signaling, see Fig. 1.3 (simplified signaling map relevant to highlight the measured proteins in my study).

AMPK isoforms

Several isoforms exist for each AMPK subunit in mammals due to whole genome duplication events (WGD): sudden evolutionary important events that result in doubling the entire genome (Berthelot et al., 2014). In humans, two genes encode the α subunit (α1 and α2 isoforms) and the β subunit (β1 and β2 isoforms) and 3 genes encode the γ subunit (γ1, γ2 and γ3 isoforms) (Craig et al., 2018; Hardie, 2018). The seven genes can provide up to 12 potential combinations of AMPK with different tissue distribution, subcellular locations and functions (Hardie, 2018; Jeon, 2016; Mihaylova and Shaw, 2011). In addition to the two WGD events that affected all bony vertebrates, salmonids

experienced a 3rd teleost-specific and a 4th salmonid-specific WGD (ssWGD) event (Berthelot et al., 2014). Recently, new genes encoding each of the three AMPK subunits were identified as a result of the ssWGD. Phylogenetic analyses found that salmonids have at least fifteen genes encoding for AMPK including four AMPK α, four AMPK β and seven AMPK γ subunits (Causey et al., 2019).

AMPK and alanine

AMPK plays a key role in regulating skeletal muscle protein metabolism through the control of protein synthesis and degradation. Under conditions of low energy such as calorie restriction and exercise for instance, AMPK promotes protein degradation to supply amino acids which can be used to provide the needed energy (Kjøbsted et al., 2018). Alanine was found to be the predominant amino acid released from peripheral tissues during overnight fasting or starvation in humans (Felig, 1973; Felig et al., 1970) The release of alanine from muscle was suggested to be the rate-limiting step for gluconeogenesis in the liver (glucose-alanine cycle) during starvation (Felig et al., 1970). Interestingly, muscle AMPK was found to be essential in the regulation of autophagy and glucose homeostasis in mice during prolonged fasting, specifically through controlling the glucose-alanine cycle (Bujak et al., 2015). The knockout of muscle AMPK hindered autophagy and protein breakdown which resulted in reduced serum alanine and blood glucose levels (Bujak et al., 2015). Recently, another group found that among the 20 amino acids screened, alanine acutely activated AMPK in hepatic cells and in mice liver (Adachi et al., 2018). These researchers found that alanine reduced glucose levels *in vivo* when oral or intraperitoneal glucose was administered to mice; however, alanine effects were modestly reduced when AMPK

activity was reduced in mice expressing a dominant negative AMPK mainly in skeletal muscle (Adachi et al., 2018). Together these studies suggest that alanine acts as a metabolic signal for AMPK activation in mice.

In salmonids, the white muscle represents the primary source of amino acids under conditions of calorie restriction and exercise. For instance, white muscle protein reserves decreased by 50% after five months of starvation and exercise in rainbow trout (French et al., 1981) and by 70% after spawning migration of sockeye salmon (Mommsen et al., 1980). Moreover, the rate of gluconeogenesis from amino acids increased after starvation and exercise in trout (French et al., 1981). Similar to mammals, alanine seems to be the major amino acid released from white muscle when proteolysis increases in fish (French et al., 1983; Milligan, 1997; Mommsen et al., 1980). Interestingly, a higher alanine aminotransferase activity was suggested to be linked to enhanced catabolism, during muscle proteolysis induced by starvation for instance (Sánchez-Muros et al., 1998). Additionally, AMPK gene expression and activity were found to increase by sustained exercise in trout muscle (Magnoni et al., 2014; Morash et al., 2014). AMPK transcript levels were reported to remain high in salmon muscle during migration, but mRNA levels decreased before spawning (Morash et al., 2013). These studies suggest that there might be an interplay between alanine and AMPK in fish. However, there is no evidence showing a direct link yet.

Goal of thesis

The main objective of this thesis is to improve our understanding of the role of the amino acid alanine on glucose metabolism in adult rainbow trout. Therefore, exogenous alanine was continuously infused for 4h to integrate organismal (first part) and molecular (second part) information regarding alanine's effects on glucose metabolism (Chapter 2). The goal of the first experiments of Chapter 2 was to determine whether alanine modifies glucose fluxes, R_t, R_a and R_d. Existing literature is controversial because some studies support the role of alanine as a gluconeogenic precursor (Canals et al., 1992; Cowey et al., 1977b) whereas others do not (French et al., 1981; Kirchner et al., 2003a). Other evidence suggests that alanine is preferentially used as an oxidative substrate rather than being converted to glucose in trout hepatocytes (French et al., 1981; Pereira et al., 1995). Therefore, two alternative hypotheses are proposed here to get insights as to whether and how alanine modulates glucose kinetics in trout. I anticipate that R_a glucose and glycemia will increase if alanine is mainly used as a gluconeogenic precursor (hypothesis 1). On the other hand, I anticipate that R_a and R_d glucose will be reduced if alanine is preferentially used as an oxidative fuel to partially replace glucose (hypothesis 2). The aim of experiments of the second part (Chapter 2) was to assess alanine's effects on key elements of glucose metabolism at the molecular level in liver and muscle to identify potential mechanisms that could explain the observed changes in glucose fluxes. Thus, key genes in glucose transport, glycolysis and gluconeogenesis were assessed at the expression and/or activity levels. An additional goal of the second part was to investigate if alanine activates AMPK in rainbow trout as it was recently shown in mammals (Adachi et al.,

2018). Consequently, protein levels of total and phosphorylated AMPK were quantified in addition to AKT and two TOR targets, S6 and 4EBP1, as these proteins are known to interact with AMPK (see Fig. 1.3). Finally, the results were compared to findings from literature and discussed in an applied context (Chapter 3). Correlative analyses were then performed to determine potential correlations between plasma alanine and glucose levels and the measured molecular indices, such as the expression and activity of the measured enzymes in the glycolytic and gluconeogenic pathways or signalling proteins quantified in tissues.

Table 1.1. Protein requirement[a] (as % of diet) for several growing and adult animals.

Species	Young	Adult
Dog	12	4
Rat	12	4.2
Man	8	5
Cat	29	19
Mink	31	20
Fox	24	16
Rainbow trout	42-48	35-40

[a]All essential amino acid requirements were met.

Adapted from (Hardy, 2002; MacDonald et al., 1984)

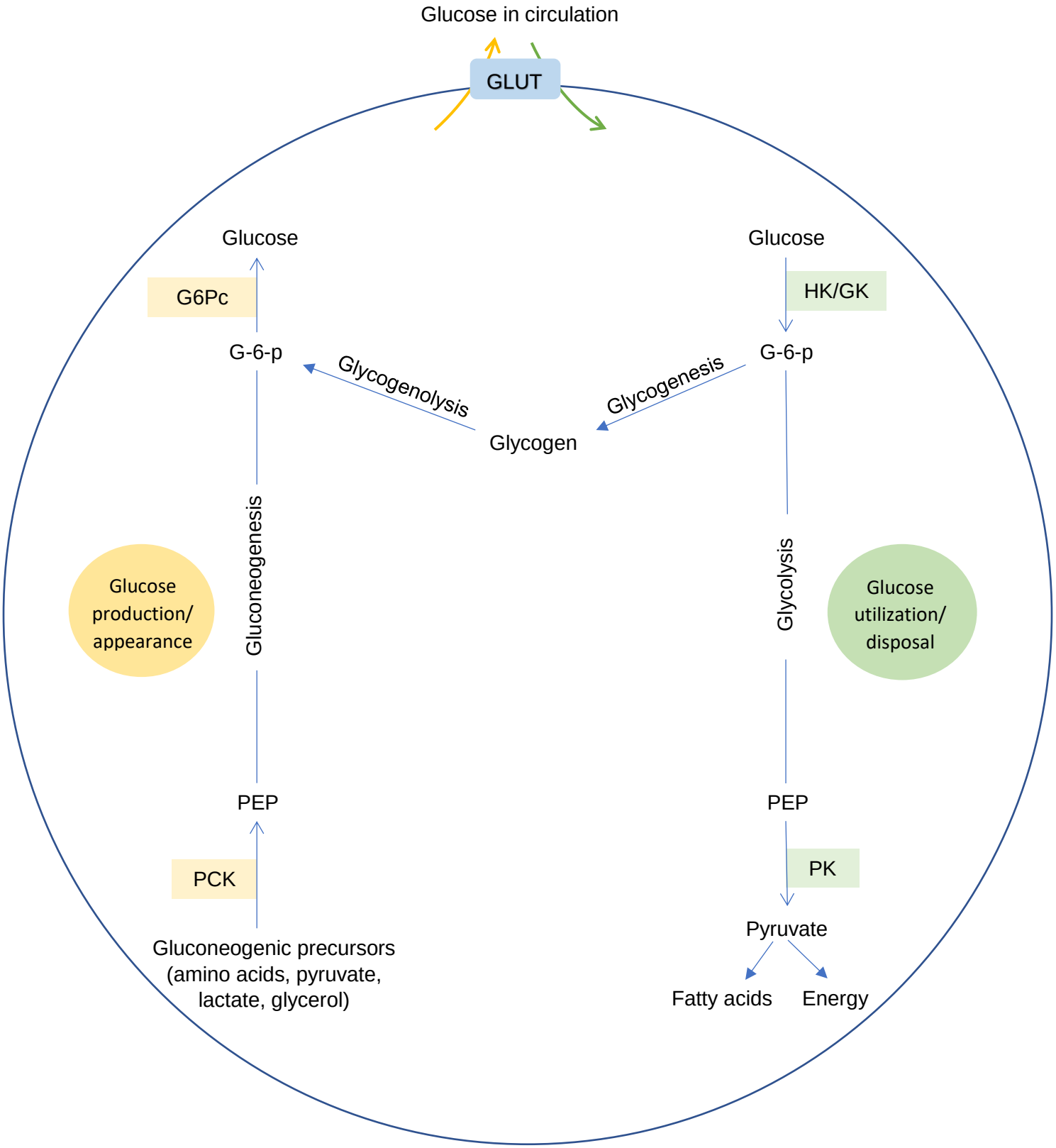
Glucose in circulation
GLUT
Glucose
G6Pc
G-6-p
Glycogenolysis
Glycogenesis
Glycogen
Glucose
HK/GK
G-6-p
Gluconeogenesis
Glycolysis
Glucose production/ appearance
Glucose utilization/ disposal
PEP
PCK
Gluconeogenic precursors (amino acids, pyruvate, lactate, glycerol)
PEP
PK
Pyruvate
Fatty acids
Energy

Fig. 1.1. Schematic representation of the glycolytic and gluconeogenic pathways. Key enzymes of the first and last steps in glycolysis are hexokinase/glucokinase (HK/GK) and pyruvate kinase (PK), respectively. The first and last steps in gluconeogenesis are catalyzed by phosphoenolpyruvate kinase (PCK) and glucose-6-phosphatase (G6Pc), respectively. The other acronyms, GLUT, G-6-P and PEP mean glucose transporter, glucose-6-phosphate and phosphoenolpyruvate, respectively. Adapted from (Enes et al., 2009).

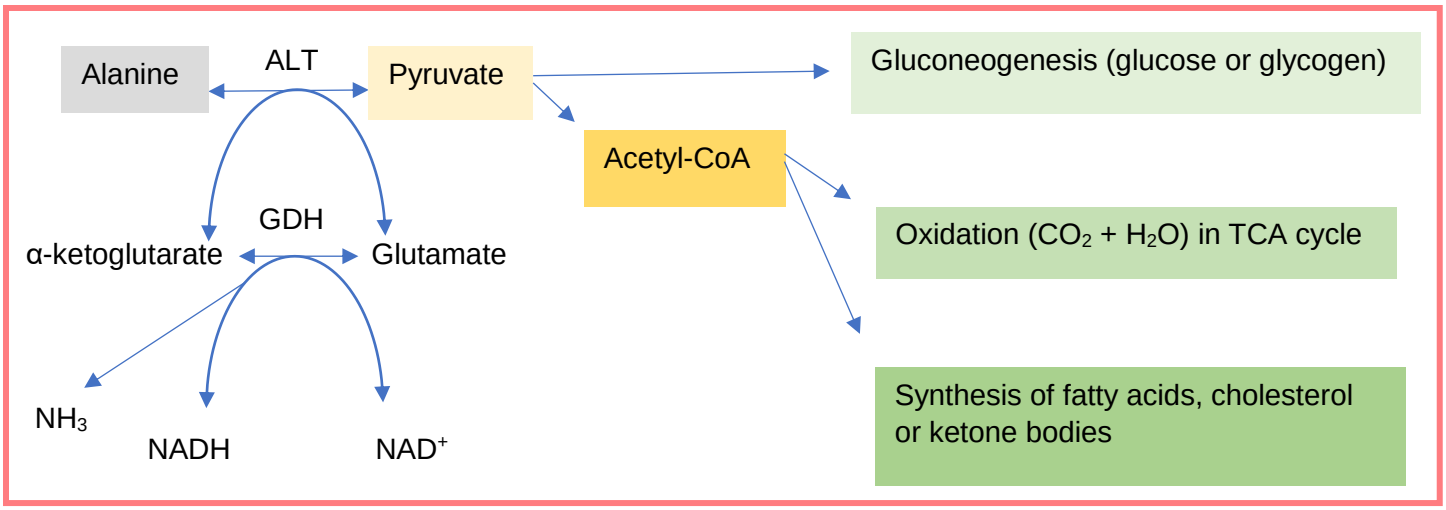

Fig. 1.2. Transamination and deamination. Alanine can be converted to pyruvate by alanine aminotransferase (ALT) which transfers the amine group from alanine to α-ketoglutarate to form pyruvate and glutamate. Pyruvate can be converted to glucose (or glycogen) by gluconeogenesis or to acetyl-CoA. The latter can be either oxidized in the TCA cycle or used for the synthesis of other substrates. The deamination of glutamate is catalyzed by glutamate dehydrogenase (GDH) which generates ammonia (NH_3) and α-ketoglutarate. Adapted from (Blanco and Blanco, 2017; Mommsen et al., 1980).

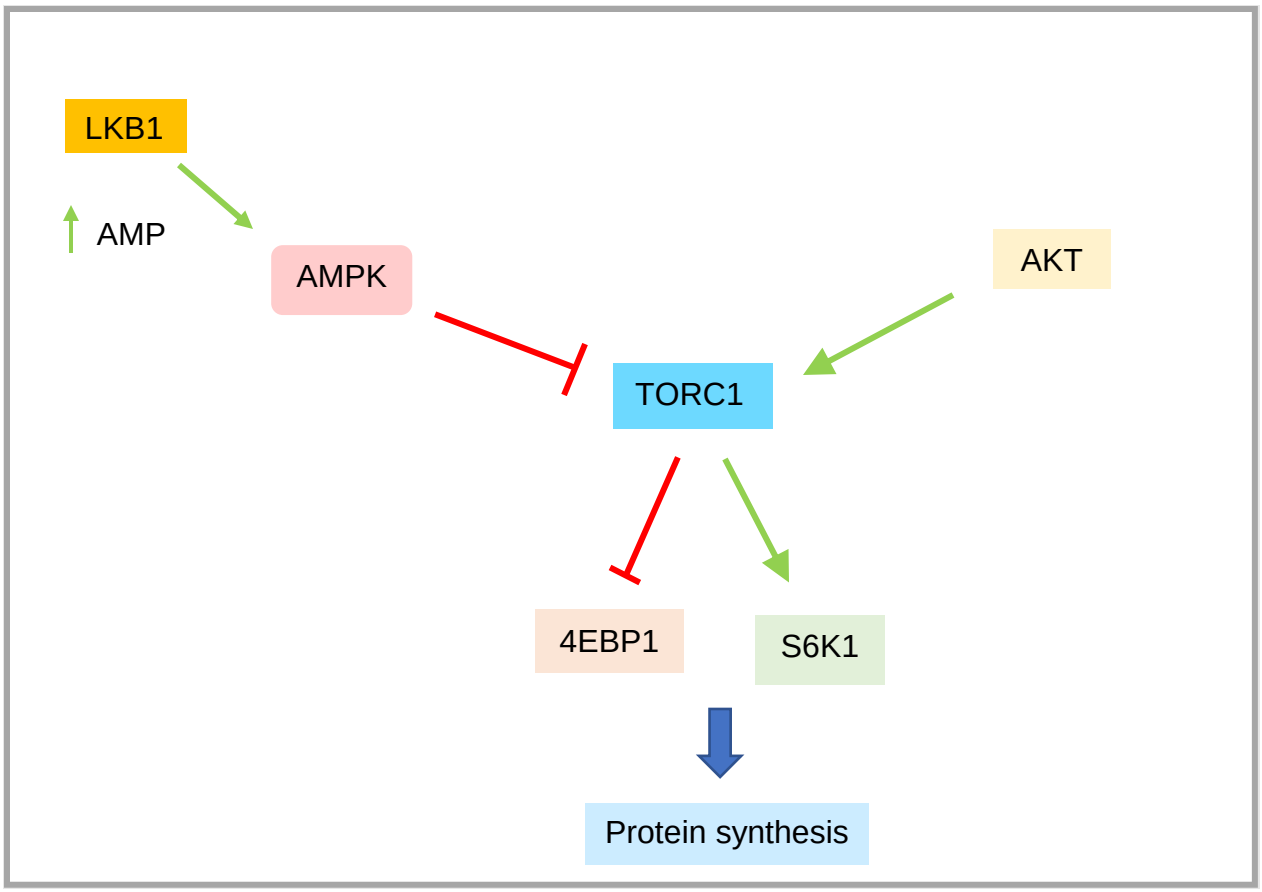

Fig. 1.3. A simplified diagram of the Adenosine monophosphate-activated protein kinase (AMPK) pathway. The interaction of AMPK with the target of rapamycin complex 1 (TORC1) and protein kinase B (AKT) is depicted. LKB1: liver kinase B1, 4EBP1: eukaryotic initiation factor 4E binding protein 1 and S6K1: ribosomal protein S6 kinase 1. Adapted from (Cornu et al., 2013; Kjøbsted et al., 2018; Mihaylova and Shaw, 2011).

Chapter 2: Alanine alters carbohydrate metabolism of rainbow trout: Glucose fluxes and cell signaling

Based on a manuscript by the same title

Witten by

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Introduction

Glucose is a key metabolic fuel and the preferred substrate of many tissues in mammals (Polakof et al., 2011c). In carnivorous trout, dietary carbohydrates are poorly metabolized and only represent a minor energy source compared to proteins and lipids (Polakof et al., 2012). However, current evidence suggests that glucose is still essential for trout, especially in the brain (Polakof et al., 2012). Understanding the metabolic limitations of glucose utilization is needed to develop sustainable and less costly carbohydrate-rich, plant-based feeds for important aquaculture species (Panserat et al., 2019). One prevalent hypothesis suggests that high levels of dietary amino acids are responsible for the slow regulation of carbohydrate metabolism observed in trout (Panserat et al., 2013).

How fish adjust their glucose fluxes to regulate glycemia can be assessed by tracer methods that allow to measure the rate of steady-state glucose turnover (R_t), or the rates of glucose appearance in (R_a) and disposal from the circulation (R_d) separately, under non-steady-state conditions (Haman and Weber, 1996). Even though some information is already available on the regulation of trout glucose fluxes (Forbes et al., 2019a; Forbes et al., 2019b; Weber et al., 2016), the effects of amino acids on *in vivo* glucose kinetics are unknown. Alanine is generally considered an important gluconeogenic precursor (Felig, 1973; Jürss and Bastrop, 1995). In mammals, exogenous alanine activates gluconeogenesis, but it fails to affect R_a glucose (Diamond et al., 1988; Jahoor et al., 1990; Wolfe et al., 1987). The extra glucose produced from alanine appears to be predominantly channeled towards hepatic glycogen rather than released in the circulation. The potential effects of alanine on trout gluconeogenesis are

less clear because activation of the pathway has been reported *in vivo* (Cowey et al., 1977b) and for isolated hepatocytes (Canals et al., 1992), but some studies show no stimulation of gluconeogenic enzymes (Kirchner et al., 2003a) or suggest that alanine is not an important precursor for glucose (French et al., 1981). Alternately, amino acids can be significant oxidative substrates for carnivorous fish (Ballantyne, 2001) and trout hepatocytes prefer to oxidize alanine rather than to convert it to glucose (French et al., 1981; Pereira et al., 1995). Therefore, it is conceivable that exogenous alanine could partly replace glucose as an oxidative fuel.

Recent evidence shows that alanine also acts as a signaling molecule because it stimulates AMP-activated protein kinase (AMPK) in mammals (Adachi et al., 2018). A high AMP/ATP ratio activates AMPK via phosphorylation, thereby stimulating catabolic pathways (to restore normal ATP levels) and inhibiting anabolic pathways such as protein synthesis via target of rapamycin (TOR)(Hardie, 2018). AMPK activity is regulated by insulin and nutrient availability through inhibition by AKT (also known as protein kinase B)(Jeon, 2016). In mice, alanine activation of AMPK via alanine aminotransferase (ALT) inhibits downstream TOR effectors and promotes glucose utilization (Adachi et al., 2018). Current knowledge of how AMPK (Polakof et al., 2011a) and TOR (Lansard et al., 2010) affect trout metabolism suggests a conserved role for these signals. However, it is not known whether alanine acts on AMPK, AKT and TOR in trout in the same way as it does in mammals (Adachi et al., 2018).

The aim of the present study was to integrate organismal and molecular information to characterize the effects of exogenous alanine on trout glucose metabolism. Our first goal was to measure whether alanine modulates R_t, R_a and/or R_d glucose. Current

literature suggests two alternative hypotheses about how glucose fluxes could be affected. If exogenous alanine acts mainly as a gluconeogenic precursor, R_a glucose and glycemia will both increase (hypothesis 1). Alternately, if alanine is predominantly used as an oxidative fuel, it will replace some glucose and reduce R_a and R_d (hypothesis 2). The second goal of this study was to explore potential mechanisms responsible for the observed changes in glucose fluxes. Therefore, the expression of key genes involved in glucose transport, glycolysis, and gluconeogenesis, as well as the activities of essential enzymes were measured in muscle and liver. Protein levels of AMPK, AKT and two TOR targets, ribosomal protein S6 (S6) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), were also quantified. This allowed to test whether alanine activates AMPK signaling in rainbow trout as recently shown in mammals (Adachi et al., 2018).

Material and methods

Animals

Adult rainbow trout (Oncorhynchus mykiss) of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). The fish were maintained in 1,200-liter flow-through tanks supplied with dechlorinated Ottawa tap water at 13°C and were exposed to a 12 h:12 h light:dark photoperiod. They were acclimated to these conditions for a minimum of 2 weeks before experiments. Trout were fed Orient LP diet (Skretting, New Brunswick, Canada) 5 days/week. The diet contains 39% protein, 16% fat and 3% fiber. This study consisted of two groups of fish; the first was used for *in vivo* measurements of glucose kinetics by continuous tracer infusion and the second

involved measurements at the molecular level by real-time RT-PCR, Western blots and enzyme activity for key indices of glucose metabolism (physical characteristics of the experimental groups are given in Table 2.1). All procedures were approved by the Animal Care Committee of the University of Ottawa and complied with the guidelines established by Canadian Council on Animal Care (CCAC).

Cannulations

Fish were anesthetized using ethyl 3-aminobenzoate methanesulfonate (60 mg/l MS-222 buffered with 0.2 g/l sodium bicarbonate) and cannulated with BTPE-50 catheters (Instech Laboratories, Plymouth Meeting, PA, USA) in the dorsal aorta as described previously (Haman and Weber, 1996). Fish used for glucose kinetics were doubly cannulated and those used for the molecular experiments were singly cannulated as labeled glucose was not infused in the latter. Cortland saline containing 50 U/ml heparin (Sigma-Aldrich, St Louis, MO, USA) was used to flush the catheters after being placed in the dorsal aorta and 25 U/ml of heparin in Cortland saline was used during blood sampling to prevent clotting. For the glucose kinetics experiments, alanine or Cortland saline (for the control group) and labeled glucose were infused through one catheter and blood was sampled through the second. For the molecular experiments, blood samples were taken before the infusion and at the end of the experiment and alanine or Cortland saline were infused through one catheter. Fish were kept in a 90-liter swim tunnel respirometer (Loligo Systems, Tjele, Denmark) supplied 13°C aerated and dechlorinated Ottawa tap water to recover overnight before the infusion. All experiments were conducted in resting fish at a water velocity of 0.5 body length/s.

Glucose kinetics experiments

The catheters were made accessible through the swim tunnel lid by channeling them through a water-tight port. Fish (N=9 for the alanine group and 7 for controls) were continuously infused with [6-^{3}H] glucose (2220 GBq/mmol; Perkin Elmer, Boston, MA) to measure R_t, R_a and R_d glucose. The infusate was prepared for each fish immediately before infusion by drying 20 µl of the tracer solution under N_2 and resuspending in 10 ml of Cortland saline. A priming dose of the tracer, equivalent to 90 min of infusion, was injected as a bolus to reach isotopic steady-state in <45 min (Shanghavi and Weber, 1999). Thereafter, the tracer was infused at ~1 ml/h (finely adjusted for each fish to account for differences in body mass) using a calibrated syringe pump (Harvard Apparatus, South Natick, MA). Labeled glucose infusion rate averaged 2149±346 Bq kg^{-1} min^{-1} (N=9) for the alanine-infused fish and 1676±135 Bq kg^{-1} min^{-1} (N=7) for the control fish. These trace amounts of labeled glucose accounted for <0.00001% of the baseline rate of hepatic glucose production in trout (Shanghavi and Weber, 1999). The difference in labeled glucose infusion rates between the alanine and the control groups does not influence glucose fluxes because if there is an increase in infusion rate, there is similarly an increase in glucose specific activity. The infusion rate and specific activity are both used to calculate glucose fluxes (see page 8 for the equations). Blood samples (~100 µl each) were taken 50, 55 and 60 min after the start of infusion to establish baseline glucose kinetics and then every 30 min during the alanine (Sigma -Aldrich A7627; at a rate of 443 µg.kg^{-1}.min^{-1}) or Cortland saline infusions that lasted 4 h. The alanine infusion rate was selected on the basis of a previous study (Robinson et al., 2011) and on pilot experiments. The amount of blood drawn from each fish accounted

for <10% of total blood volume. Blood samples were collected in tubes containing heparin and aprotinin, and they were immediately centrifuged for 5 min at 12,000 rpm (Eppendorf 5415C, Brinkman, Rexdale, Canada). Plasma was separated and stored at −20°C until analyses.

Plasma sample analyses

Plasma alanine and glucose concentrations were measured spectrophotometrically using a Spectra Max Plus 384 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Plasma alanine concentration was measured using an EnzyChrom L-Alanine Assay Kit (BioAssay Systems, USA). Glucose concentration was determined using an enzymatic assay that measures the absorbance of NADH at 340 nm with 7 U/ml hexokinase (H4502, Sigma) and 0.1 U/ml glucose-6-phosphate dehydrogenase (G5885, Sigma) in a buffer containing 59.84 mM Trizma base, 39.98 mM Tris-HCl, 1.0142 mM $MgSO_4.7H_2O$, 2.215 mM NAD^+ and 1.106 mM ATP. For the glucose kinetics experiments, radioactivity was measured by scintillation counting (Beckman Coulter LS 6500, Fullerton, CA) by drying 20 µl of plasma under nitrogen gas to eliminate tritiated water, resuspending in 1 ml of deionized water and adding 10 ml of Bio-Safe II scintillation cocktail (RPI, Mount Prospect, IL).

Molecular experiments

These experiments were carried out on different fish than those used for the glucose kinetics to avoid handling radioactivity unnecessarily. The same rate of alanine (alanine group, N=7; 443.2 µg alanine.kg^{-1}.min^{-1}) or Cortland saline infusion (control group, N=7) was administered at ~1 ml/h for 4 h. A baseline blood sample was taken at

time 0 h before the start of alanine/saline infusion and at 4 h. The fish were then euthanized by a sharp blow on the head and tissues were collected. Whole liver, white muscle (perpendicular to the dorsal fin) and red muscle (along the lateral line) were collected and stored at –80°C until analyses.

RNA extraction and gene expression

Total RNA from white and red muscle was extracted by homogenizing 50 mg of tissue in 1.5 ml tubes using a sonicator (Fisher Scientific Sonic Dismembrator model 100, San Diego, CA, USA) and Trizol reagent (life technologies). RNA was quantified using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo-Fisher Scientific).

QuantiTect reverse transcriptase kit (Qiagen, Toronto, ON, Canada) was used to generate cDNA using total RNA from white and red muscle tissues following the manufacturer's protocol. A no-reverse transcriptase (RT) negative control, where the RT was replaced with water, and a no-template negative control, where the RNA was replaced with water, were included to check for genomic DNA contamination.

White and red muscle mRNA gene expression of glucose transporter (*glut4a* and *glut4b*) and hexokinase (*hk2*; presented in supplementary data, Fig. S2.2) were assessed by two-step real-time RT-PCR in each tissue using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada) following the manufacturer's protocol and using the *BioRad* CFX96- Real-Time System- C1000 Thermal Cycler. The reference gene elongation factor 1α (*ef1α*) was used to normalize the mRNA abundance of the white and red muscle genes (*glut4a*, *glut4b* and *hk2*) and it has been previously used in rainbow trout (Kostyniuk et al., 2019a; Polakof et al.,

2011a). Liver mRNA gene expression (presented in supplementary data, Fig. S2.1 and Fig. S2.2) of key gluconeogenic genes (phosphoenolpyruvate carboxykinase, *pck1*, *pck2a*, *pck2b*, and glucose-6-phosphatase, *g6pca*, *g6pcb1a*, *g6pcb2a*, *g6pcb1b* and *g6pcb2b*) and glycolytic genes (glucokinase, *gka* and *gkb*) were assessed similarly using iTaq Universal SYBER Green SuperMix (Bio-Rad). *β-actin* mRNA abundance was used as a reference gene for the liver and it has been previously used in trout (Moltesen et al., 2016). Standard curves from pooled cDNA serial dilution were generated to optimize/check reaction conditions. Diluted samples were run in duplicate. The total volume of the reaction was 20 µl including 1 µl cDNA, 1 µl specific forward primer, 1 µl specific reverse primer (Table S2.1), 10 µl Universal SYBR Green Supermix and 7 µl nuclease free water. An initial step at 98°C (2 min) was required for the activation of the enzymes in the mix. Then, two steps were repeated 40 times, the denaturation step at 95°C (20 s) and the annealing combined with the extension step (30 s) at the optimized temperature for each primer pair (Table S2.1). At the end, a melt step from 65°C to 95°C (0.5°C increment/5 s) was included to check the specificity of the produced amplicon by assessing the melt curve where a single melt peak indicates a specific product. The amplification efficiencies and R^2 are included in Table S2.1. The abundance of mRNA was calculated relative to the control group.

Western blotting

Total protein was extracted from frozen liver and muscle tissue by homogenizing 100 mg of tissue in 400 ul of buffer on ice using a sonicator (Fisher Scientific Sonic Dismembrator model 100, San Diego, CA, USA). Liver homogenization buffer (pH ~ 8) contained 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50

mM Tris HCl, 1 mM EDTA, 100 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate and a protease inhibitor cocktail (A32953, Thermo Scientific). Muscle tissues were homogenized in a buffer as previously described (Forbes et al., 2019a; Forbes et al., 2019b). Homogenates were centrifuged at 15,000 g for 10 min at 4°C, and the supernatant was taken and stored at -20°C. Protein concentrations were determined using bicinchoninic acid (BCA) assay (B9643, Sigma) with bovine serum albumin (BSA) as a standard. Protein samples were denatured and reduced before being loaded into the gels. Protein samples were diluted in equal amount of the above-described buffer and 2X Laemmli buffer for a total amount of 25 µg protein in 20 µl of loading volume. The samples were denatured at 95°C for 5 min and quickly placed on ice. To load protein samples, gels were casted as a 10% resolving gel consisting of 5 ml ddH_2O, 2.5 ml buffer B (1.5 M Tris base and 0.04% SDS dissolved in dH_2O, pH 8.8), 2.5 ml 40%Acryl/Bis (Bio-Rad), 50 µl 10% ammonium persulfate (APS) (A3678, Sigma) and 20 µl TEMED (Invitrogen); and a 4% stacking gel consisting of 3.25 ml ddH_2O, 1.25 ml buffer C (0.5 M Tris and 0.04% SDS dissolved in dH_2O, pH 6.8), 0.5 ml 40%Acryl/Bis, 25 µl 10% APS and 10 µl TEMED. Gels were placed in 1X Tris glycine SDS (TGS) running buffer consisting of 2.5 mM Tris base, 0.192 M glycine and 0.1% SDS dissolved in dH_2O. Protein samples were loaded with 5 µl of Page Ruler prestained protein ladder (26616, Thermo Scientific, Lithuania). The proteins were migrated for ~2 h at 100 V using the Mini Protean Tetra Cell (Bio-Rad, China). Immediately after migration, the gels were blotted for ~2 h at 100 V on nitrocellulose membrane (pore size 0.2 µm, Bio-Rad, Germany) in 20% Western transfer buffer consisting of 250 mM Tris base, 1.92 M glycine dissolved in dH_2O. Then, the

membranes were incubated in Odyssey blocking buffer (LI-COR) for 1 h at room temperature on an orbital shaker. Membrane were incubated overnight at 4°C in rabbit-raised primary antibodies diluted to 1:1000 in the blocking buffer specific for p-AKT (S473; no. 9271S) and total AMPKα (no. 2532) all from Cell Signaling Technology. AKT (Forbes et al., 2019a) and AMPKα (Gilmour et al., 2017; Polakof et al., 2011a) antibodies were previously used in rainbow trout. The phosphorylated form of AMPKα (T172, no. 2535S), and two TOR targets, S6 (S235/236, no. 4856S) and 4EBP1 (T37/46, no. 9459S), were also quantified in liver and muscle (see supplementary data). Membranes were then washed 4 times for 5 min with phosphate buffered saline (PBS) + 0.1% Tween 20 (P9416, Sigma) and incubated with IRDye 800 CW goat-anti-rabbit secondary antibody (925-32211, LI-COR) diluted to 1:5000 in the blocking buffer for 1.5 h at room temperature on the shaker protected from light. Membranes were then washed 4 times for 5 min with PBS + 0.1% Tween 20 and 1 time for 5 min with PBS and visualized by infrared fluorescence using the Odyssey Imaging System (LI-COR) and band intensity was quantified by Odyssey Infrared imaging system software (LI-COR). β-tubulin (no. 2146S, Cell Signaling Technology) was visualized and quantified to normalize the measured protein intensities and it has been previously used in rainbow trout to normalize protein levels (Forbes et al., 2019a; Polakof et al., 2011a).

Enzyme activities

Liver tissue (1:4 w/v), red and white muscle tissue (1:16 w/v) were sonicated on ice in cold 50 mM Tris buffer pH 7.5 with protease inhibitor mini tablets (A32953, Thermo Scientific) as previously described (Best et al., 2014; Ings et al., 2011). Whole homogenate was used. Aliquots were prepared by diluting the homogenate in 50%

glycerol buffer pH 7.5 (50% glycerol, 21 mM Na_2HPO_4, 0.5 mM EDTA-Na_2 dihydrate, 0.2% BSA, 5 mM β-mercaptoethanol) and stored at -20°C. Enzyme activity was determined kinetically at 22°C by measuring the reduction of NADH at 340 nm using a microplate reader. Enzyme activity was expressed as µmol/min/g tissue. The activity was assayed in triplicate and the conditions of the individual enzyme assay were as detailed below (final concentration in the well; all reagents dissolved in 50 mM imidazole buffer pH 7.4). Hexokinase (HK; EC 2.7.1.1) assay mixture consisted of 1 mM glucose, 5 mM $MgCl_2$, 0.24 mM NADH, 2 mM phosphoenolpyruvate (PEP), 20 U/ml lactate dehydrogenase (LDH), 5 U/ml pyruvate kinase (PK) and 4 mM ATP (to start the reaction). Glucokinase (GK; EC 2.7.1.2) assay mixture contained 20 mM glucose, 5 mM $MgCl_2$, 0.24 mM NADH, 2 mM PEP, 20 U/ml LDH, 5 U/ml PK and 4 mM ATP (to start the reaction). Phosphoenolpyruvate carboxykinase (PCK; EC 4.1.1.32) reaction mixture contained 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM PEP (Na_3), 0.12 mM NADH and 0.2 mM deoxyguanosine diphosphate (dGDP; to start the reaction). Alanine aminotransferase (ALT; EC 2.6.1.2) reaction mixture contained 0.12 mM NADH, 200 mM L-alanine, 0.025 mM pyridoxal 5-phosphate, 12 U/ml LDH (no ammonium sulphate) and 10.5 mM α-ketoglutarate (to start the reaction).

Calculations and statistics

Fulton's condition factor was calculated using the equation $K= (10^5 \times M_b)/L^3$, where M_b is body mass in g and L is length in mm (Blackwell et al., 2000). Steady-state and non-steady-state equations of Steele were used to calculate glucose fluxes in two ways (Steele, 1959). The steady-state equation was used to calculate R_t glucose. R_a and R_d glucose were calculated separately using the non-steady-state equations

(Forbes et al., 2019a; Steele, 1959) using glucose specific activities curve-fitted by second-degree polynomial regression for each fish individually (Wolfe, 1992). Statistical analysis for glucose kinetics was performed using one-way repeated-measures analysis of variance (RM-ANOVA) with Dunnett's post hoc test to determine which time point was statistically different from the average baseline value using SigmaPlot v12.5 (Systat Software, San Jose, CA, USA). Two-way RM-ANOVA was used to analyze the non-steady-state data where time and R_a or R_d were the 2 factors. When the assumption of normality or equal variances were not met, data were transformed to their ln or square root. Kruskal-Wallis non-parametric one-way ANOVA on ranks was used when transformation failed to meet the assumptions of parametric tests. Molecular data (gene expression, signaling and enzyme activity) were analyzed by two-tailed unpaired t-tests using GraphPad Prism 8.3.1. Data were checked for normality using the Shapiro-Wilk test. When the normality assumption was not met, data were transformed (ln or sin transformations). The Grubb's test was used on normally distributed data to check for outliers. When variances of the two groups, control and alanine, were not equal, Welch's test was used for correction. Mann-Whitney non-parametric test was applied when the normality assumption was not met after transformation. Glucose and alanine concentrations were analyzed by two-way RM ANOVA. The mean + s.e.m. (± s.e.m. for glucose kinetics) are shown and $P<0.05$ was considered significant in all tests.

Results

Plasma alanine, glycemia and glucose kinetics

The effects of exogenous alanine infusion on plasma alanine levels, glucose concentration and specific activity are presented in Fig. 2.1. The infusion of exogenous alanine caused a significant increase in plasma alanine concentration (*p=0.04*; Fig. 2.1A), specifically at 0.5, 3 and 4 h compared to baseline. Alanine increased from an average baseline value (0 h) of 0.6±0.1 µmol ml^{-1} to a final value (4 h) of 3.0±0.8 µmol ml^{-1}. Alanine administration caused a decrease in glucose concentration after 2 h (*p<0.001*; Fig. 2.1B). Glucose levels decreased from a baseline value of 8.7±0.3 µmol ml^{-1} to a final value of 7.3±0.7 µmol ml^{-1}. Glucose specific activity increased after 3 h of exogenous alanine infusion (*p=0.003*; Fig. 2.1C). Glucose specific activity increased from a baseline value of 197.4±42.9 Bq μmol^{-1} to a final value of 373.0±83.4 Bq μmol^{-1}.

The effect of alanine on glucose flux is presented in Fig. 2.2 and Fig. 2.3. R_t glucose decreased over time (*p<0.001*; Fig. 2.2A) from a baseline value of 14.9±2.7 µmol kg^{-1} min^{-1} to a final value of 6.8±0.9 µmol kg^{-1} min^{-1}. Similarly, hepatic R_a and R_d glucose decreased over the 4 h of alanine infusion (*P<0.01*; Fig. 2.3). R_a glucose decreased from a baseline value of 13.2±2.5 µmol kg^{-1} min^{-1} to a final value 7.3±1.6 µmol kg^{-1} min^{-1}. R_d glucose decreased from a baseline value of 13.2±2.5 µmol kg^{-1} min^{-1} to a final value of 7.4±1.5 µmol kg^{-1} min^{-1}. R_d glucose is slightly higher than R_a glucose as it can be seen in Fig. 2.3.

Plasma alanine and glucose levels as well as glucose specific activity were measured in the control fish infused with Cortland saline and they are presented in Fig.

2.1. All the measured parameters did not change from the average baseline (*p>0.05* for all the parameters; Fig. 2.1D, E and F). R_t glucose did not show significant change over time compared to the average baseline, but the data showed a decreased trend *(p>0.05*; Fig. 2.2B). When the lowest point (at time -5 min) was removed from the average baseline, there was a significant decrease ($p<0.05$) over time in R_t glucose in the control group. However, this point is not an outlier. It is unclear what causes this change.

Plasma alanine and glucose for molecular experiments

Baseline and final plasma alanine and glucose concentrations in the alanine and control groups are presented in Table 2.2. Alanine levels increased after 4 h of exogenous alanine administration (*p=0.002*) and compared to the control group (*p=0.0006*). The interaction between the time and treatment was significant (*p=0.002*). Plasma glucose concentration decreased after 4 h of exogenous alanine infusion regardless of the treatment, Cortland saline or alanine (*p=0.03*).

Gene expression

The effect of alanine on red and white muscle *glut4a* and *glut4b* mRNA abundance is shown in Fig. 2.4. The relative abundance of red muscle *glut4b* decreased in the alanine group (*p=0.01*; Fig. 2.4), but the mRNA abundance of the other genes was unaffected by the treatment (*p>0.05*; Fig. 2.4). The mRNA abundance of the paralogues of two gluconeogenic genes, *pck* and *g6pc*, was assessed in the liver as shown in Fig. S2.1. Alanine had no effect on the transcript abundance of *pck* paralogues (*p>0.05*; Fig. S2.1A). Among the five paralogues of *g6pc*, only *g6pcb1b*

increased in the alanine group (*p=0.04*; Fig. S2.1B). The transcript abundance of the glycolytic genes *gka* and *gkb* in the liver and *hk2* in red and white muscle is presented in Fig. S2.2. Alanine had no effect on the mRNA abundance of these genes in the liver, white and red muscle (*p>0.05*; Fig. S2.2).

Protein signaling

The effect of alanine on the relative level of total AMPKα was assessed by Western blotting in the liver as shown in Fig. 2.5. After 4 h of exogenous alanine administration, total AMPKα increased in the alanine group (*p=0.02*; Fig. 2.5). The level of phosphorylated AKT was quantified in the liver and red muscle as presented in Fig. 2.6. Alanine had no effect on liver p-AKT, but there is a trend of a decrease in the alanine group (*p=0.06*; Fig. 2.6). However, the level of red muscle p-AKT increased in the alanine group after 4 h of exogeneous alanine infusion (*p=0.03*; Fig. 2.6). The relative level of phosphorylated AMPKα and two TOR targets, S6 and 4EBP1, was assessed in the liver (Fig. S2.3) and red muscle (Fig. S2.4). Alanine had no effect on the phosphorylated form of these proteins in the liver (*p>0.05*; Fig. S2.3) and red muscle (*p>0.05*; Fig. S2.4).

Enzyme activities

Enzyme activity of ALT, GK and PCK in the liver and HK in the red and white muscle is shown in Fig. 2.7. Alanine had no significant effect on the activity of the measured enzymes in liver (*p>0.05*; Fig. 2.7). Hexokinase activity decreased in both red and white muscle (*p=0.007* and *p=0.02* respectively; Fig. 2.7).

Discussion

This study is the first to show that alanine reduces glycemia in rainbow trout. This reduction is linked to decreases in R_a and, to a lesser extent, R_d glucose. At the molecular level, the alanine-dependent decrease in R_d glucose can be partially explained by a reduction in *glut4b* gene expression and HK activity in red and white muscle. Together, these findings show that alanine does not stimulate hepatic glucose release but limits peripheral glucose utilization. The reduction in glycemia observed here is consistent with recent findings in mice (Adachi et al., 2018). However, the molecular mechanisms involved appear to be different. In mice, the alanine-dependent reduction in glycemia is linked to the activation of hepatic AMPK, the stimulation of glucose uptake and reduction of its release (Adachi et al., 2018). In trout, alanine failed to stimulate hepatic p-AMPK, although total AMPK increased. However, p-AKT increased in red muscle, but not in liver, mimicking earlier findings after insulin infusion in trout (Forbes et al., 2019a). Together, present results suggest that the glucose-lowering effects of alanine is indirectly mediated by insulin in trout.

Inhibition of glucose appearance

Alanine inhibits R_a glucose in trout (Fig. 2.3), but had no such effect in humans (Jahoor et al., 1990; Wolfe et al., 1987) or dogs (Diamond et al., 1988). R_a glucose is controlled by gluconeogenesis and glycogen breakdown (glycogenolysis). The rate-limiting enzymes catalyzing the first step in gluconeogenesis and glycogenolysis are PCK and glycogen phosphorylase (GP), respectively. The last step of both gluconeogenic and glycogenolytic pathways is catalyzed by G6Pc (Enes et al., 2009). In trout, alanine affected neither *pck* expression (Fig. S2.1A) or PCK activity (Fig. 2.7), nor

g6pc expression (except for a single paralogue; Fig. S2.1B). In juvenile trout fed an alanine-rich diet over several weeks, no effect on cytosolic PCK activity and an inhibition of the expression and activity of G6Pc were found (Kirchner et al., 2003a). Despite the different experimental designs, both the current work and the dietary study by Kirchner and colleagues show that alanine does not stimulate hepatic glucose production in trout. In mammalian and avian species, alanine had no effect on PCK and G6Pc activities (Donaldson and Christensen, 1992; Friedrichs and Schoner, 1974).

Given the observed alanine-dependent reduction of R_a glucose in the absence of effects on PCK activity, it is possible that alanine inhibited hepatic glycogenolysis. Glycogen metabolism is principally regulated by phosphorylation, which elicits opposing effects on glycogen phosphorylase (activated by phosphorylation), and glycogen synthase (inhibited by phosphorylation) (Enes et al., 2009). While the hepatic expression and activity of GP and glycogen content were not measured in the current study, findings in fish and mammals suggest a role for alanine in promoting glycogen synthesis. In sea raven (*Hemitripterus americanus*) hepatocytes, insulin increased gluconeogenesis from alanine and had a small positive effect on glycogen content (Foster and Moon, 1987). In mammals, glycogen synthesis from alanine was reported (Shalwitz et al., 1989). Together, our findings refute the hypothesis that alanine increases R_a glucose. However, future studies investigating potential effects of alanine on glycogen metabolism are warranted.

Inhibition of glucose utilization

Alanine inhibits R_d glucose in rainbow trout (Fig. 2.3). The same effect was reported in humans (Jahoor et al., 1990) and a weaker but similar response was found in dogs

(Diamond et al., 1988). However, glucose utilization was unaffected by alanine *in vitro* in rat brain (Lütz et al., 2003). Glucose disposal is regulated by glucose uptake, glycolysis and glycogen synthesis. Glucose uptake is mainly mediated through GLUTs (Navale and Paranjape, 2016). The insulin-sensitive GLUT4, found mostly in muscle and adipose tissue, plays a key role in glucose disposal in mammals and trout (Díaz et al., 2007; Huang and Czech, 2007). The first step in the glycolytic pathway is catalyzed by HK in muscle and GK in liver (Enes et al., 2009). Glycogen synthase (GS) is a key enzyme in glycogenesis (Enes et al., 2009).

Alanine decreased *glut4b* in red muscle (Fig. 2.4) but did not change *hk* expression in red or white muscle (Fig. S2.2). However, HK activity decreased significantly, in both muscle types (Fig. 2.7). These reductions in *glut4b* expression and HK activity can partially explain the decrease in R_d glucose. Alanine did not affect the expression (Fig. S2.2) and activity (Fig. 2.7) of GK in the liver. Because the expression and activity of GK in trout are sensitive markers of hepatic glucose concentration (Panserat et al., 2000), this finding suggests that hepatic glucose levels were not modulated by alanine. Similarly, a 9-week dietary alanine treatment had no effect on hepatic glycolysis in juvenile rainbow trout, as indicated by a lack of changes in expression and activity of GK (Kirchner et al., 2003a). In *vitro* incubation of mammalian H4IIE hepatocytes with alanine increased glucose uptake (Adachi et al., 2018), but the amino acid did not affect GK activity *in situ* (Weber et al., 1968). Because studies on the effect of alanine (after feeding, injection or infusion) on hepatic glycolytic enzyme activity in mammals have not been reported to our knowledge, it is currently unclear whether these differences reflect methodological or true species differences.

While indices of glycogen metabolism were not quantified in our study, previous work using fish and mammalian hepatocytes suggests that exogenous alanine is metabolized to glycogen (Baquet et al., 1991; Foster and Moon, 1989; Plomp et al., 1990). It is therefore feasible that alanine may be used to support glycogen synthesis in rainbow trout. Overall, the decrease in R_d glucose suggests that preference for glucose as an oxidative fuel decreased and that it was partly replaced by alanine, although the amino acid was probably also used for glycogen synthesis. Decreased glucose import and utilization is further corroborated at the molecular level in muscle, where alanine caused reductions in the expression of glucose transporters and hexokinase activity.

Increase in total but not activated hepatic AMPK

To our knowledge, this is the first study examining the impact of alanine on AMPK in trout or any fish species. Alanine increased total AMPK in liver (Fig. 2.5) with no significant effect on p-AMPK (Fig. S2.3 and S2.4). AMPK is a heterotrimeric complex composed of 3 subunits (Jeon, 2016). It is activated when phosphorylated at threonine (Thr) 172 within the α-subunit and by binding of AMP within the γ-subunit (Craig et al., 2018). In mammals, AMPK activation by alanine inhibited TOR targets, S6 kinase and S6, and reduced glycemia *in vivo* (Adachi et al., 2018). In the current study, TOR targets, S6 and 4EBP1, were unaffected by alanine further supporting that AMPK was not activated by alanine (Fig. S2.3 and S2.4).

A differential response of AMPK activation following alanine administration may be linked to physiological and molecular differences between trout and mammals. At the physiological level, activation of hepatic AMPK in mammals was linked to increased transamination of alanine by ALT and subsequent urea synthesis, whose energetic cost

contributed to an increase in AMP levels (Adachi et al., 2018). Conversely, ALT activity was unaffected by alanine in trout liver (Fig. 2.7), in line with previous studies in trout reporting either no change (French et al., 1981) or only a small increase (Walton, 1986) in ALT activity after changes in dietary protein levels. Nitrogen waste in adult trout is mainly excreted as ammonia, while urea accounts for a small amount (Panserat et al., 2013). Ammonia-based nitrogen detoxification is energetically less costly compared to urea-based strategies (Ip and Chew, 2010). This difference in energetic cost may partially explain the lack of AMPK phosphorylation in trout. This would result in a comparatively smaller increase in AMP levels in rainbow trout, which in turn may not be high enough to trigger AMPK activation. At the molecular level, salmonid-specific whole genome duplication led to the retention of fifteen genes encoding AMPK subunits in rainbow trout, whereas only 7 genes exist in humans (Causey et al., 2019). This complexity and potential divergent regulation of paralogues may also contribute to the different responses. In contrast to the recent mammalian study (Adachi et al., 2018), our results suggest that the alanine-dependent decrease in glycemia is not mediated by AMPK activation in rainbow trout. The biological consequences of the observed increase in total hepatic AMPK are unknown but may suggest an increased capacity to respond to changes in AMP/ATP ratios.

Activation of AKT in red muscle

Alanine increased p-AKT levels in red muscle but not liver (Fig.2.6). Interestingly, this finding is consistent with results after exogenous insulin infusion in rainbow trout (Forbes et al., 2019a). In addition to the similar pattern of p-AKT stimulation following alanine and insulin infusion, the reductions in glycemia, as well as R_a and R_d glucose

were strikingly similar between the two studies. This suggests that alanine may have stimulated insulin secretion. Indeed, previous studies support the idea that alanine as well as other amino acids stimulate insulin secretion in teleost fishes, including rainbow trout (Andoh, 2007; Plisetskaya et al., 1991). Unfortunately, insulin levels cannot be readily measured in rainbow trout (Moon, 2001). Mammalian studies report different effects of alanine on pancreatic hormone release. For example, alanine increased insulin (Genuth and Castro, 1974) and glucagon (Sann et al., 1978; Wise et al., 1973) levels in humans. Interestingly, alanine can differentially regulate both hormones under different conditions in dogs (Müller et al., 1971). When glucose was available (exogenous load), alanine raised the ratio of insulin to glucagon, whereas it stimulated glucagon secretion when glucose was lacking (Müller et al., 1971). Conversely, other findings in mammals suggest alanine had no effect on both hormones (Adachi et al., 2018). In the current study, glucagon was not measured because of the limited plasma volume available. However, given that exogenous glucagon increased glycemia and R_a glucose, and decreased R_d glucose in trout (Forbes et al., 2019b), our findings suggest that alanine mediated its effects through insulin, but not glucagon in trout.

Possible fates of alanine and consequences on glucose metabolism

The concurrent reduction in R_a and R_d glucose suggests that glucose was not the preferred fuel under high alanine availability. This indicates that alanine was predominantly used as an oxidative fuel replacing glucose to some extent which supports our second hypothesis. Glucose represents a minor energy source in trout and amino acids (from dietary proteins) can be oxidized to provide energy (Panserat et al., 2013). The lack of expression and activity changes of the key gluconeogenic enzyme

PCK in this study suggests that alanine was also possibly used for glycogen synthesis via gluconeogenesis. Moreover, glycogen breakdown was probably inhibited given the reduction in R_a glucose. This likely occurred at the level of glycogen phosphorylase because a general lack of change in the expression of *g6pc* paralogues was observed. However, additional studies will be necessary to probe the specific effect of alanine on hepatic glycogen metabolism. After the conversion of alanine to pyruvate, the latter can be oxidized to provide energy (Mommsen et al., 1980). Under our conditions, energy requirements were probably met by alanine oxidation and it would be less energetically favorable to convert alanine to glucose before oxidation. In trout muscle, we speculate that alanine was preferentially oxidized over glucose because consistent reductions in molecular indices of glucose transport and glycolysis were found. Unfortunately, the fate of alanine or other amino acids in trout muscle remains largely unexplored (Weber et al., 2016).

At least in fasted trout hepatocytes, glycogenesis from alanine was preferred over gluconeogenesis, and alanine utilization was higher than glucose (Pereira et al., 1995). The production of CO_2 from alanine was higher than its conversion to glucose in trout hepatocytes indicating that alanine is a good oxidative substrate (French et al., 1981). In sea raven hepatocytes, insulin stimulated CO_2 production from alanine (Foster and Moon, 1987). In mammals, many reports found no effect of alanine on R_a glucose (Diamond et al., 1988; Jahoor et al., 1990; Wolfe et al., 1987). Thus, three fates were suggested for alanine in mammals. First, hepatic glucose produced from alanine would be converted to glycogen (Wolfe et al., 1987). Second, an excess in alanine would inhibit gluconeogenesis from other precursors; and third, alanine is possibly

preferentially used instead of glucose for energy production (Jahoor et al., 1990). The above-mentioned findings in fish hepatocytes and the fates of alanine proposed in mammals support our second hypothesis. The similar effect of both alanine (present study) and insulin (Forbes et al., 2019a) on glucose fluxes suggests that alanine probably stimulated insulin secretion which could indirectly promote alanine oxidation to provide the needed energy.

Conclusions

This is the first study that investigates the effects of alanine on glucose metabolism in trout by integrating glucose fluxes at the whole organism level and molecular indices in liver and muscle tissues. Proteins, and thus amino acids, represent the main nutritional energy source in this aquaculture species. Therefore, a better understanding of their impact on glucose metabolism is important for aquaculture. Alanine is a dominant amino acid in all tissues in rainbow trout (Storey, 1991) and has one of the highest disposal rates indicating a high requirement for alanine in this species (Robinson et al., 2011). The reduction in glucose fluxes suggests that glucose was not the preferred substrate under our conditions (high alanine availability and trout were at rest) and that alanine oxidation was probably sufficient to meet energy requirements. Mechanistically, alanine does not change molecular indices of hepatic glycolysis and gluconeogenesis raising the possibility that the reduction in R_a is linked to altered glycogen metabolism. The reduction of molecular indices related to glucose import and glycolysis in muscle tissue support decreased peripheral utilization of glucose under conditions of high alanine availability.

Comparatively, the alanine-dependent reduction in glycemia at the whole animal level is consistent with findings in mammals. However, while reduction in glycemia was mediated through AMPK activation in mammals, our data suggest that the effects of alanine do not involve AMPK activation in tout and might be indirectly mediated through insulin. Thus, different mechanisms underlie alanine's glucose-lowering effects in trout and mammals. Given the striking similarities in regulation and mechanistic underpinnings of glucose fluxes between alanine and insulin-infused trout, we hypothesize that alanine alters glucose fluxes by acting as an insulin secretagogue in this species. Future studies using insulin receptor antagonist are warranted to investigate this hypothesis.

Table 2.1. Physical characteristics and hematocrit of the two groups of rainbow trout.

	Glucose kinetics (N=16)	Molecular experiment (N=14)
Body mass (g)	463.7±16.0	412.4±14.9
Body length (cm)	33.7±0.4	33.2±0.4
Hematocrit (%)	28.7±1.9	19.2±2.1

Trout were used for (i) *in vivo* glucose kinetics measurements or (ii) tissue gene expression, signaling and enzyme activity. Fulton's condition factor *K* for all fish was 1.17±0.02 (N=30). Body mass and length were measured before the surgery. Hematocrit was measured on the second day, after recovery from the surgery to minimize blood loss. Mean values ± s.e.m. are presented.

Table 2.2. Plasma alanine and glucose concentrations for the molecular experiment.

Metabolite	Group	Baseline (0h)	Final (4h)	Time effect	Treatment effect	Time-treatment interaction
Plasma alanine ($\mu mol\ ml^{-1}$)	Control	0.5±0.1	0.4±0.1	**	***	**
	Alanine	0.8±0.1	7.4±1.7			
Plasma glucose ($\mu mol\ ml^{-1}$)	Control	7.7±0.9	5.9±0.8	*	ns	ns
	Alanine	6.6±0.7	5.7±0.7			

Values are means ± s.e.m. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns means not significant ($p>0.05$).

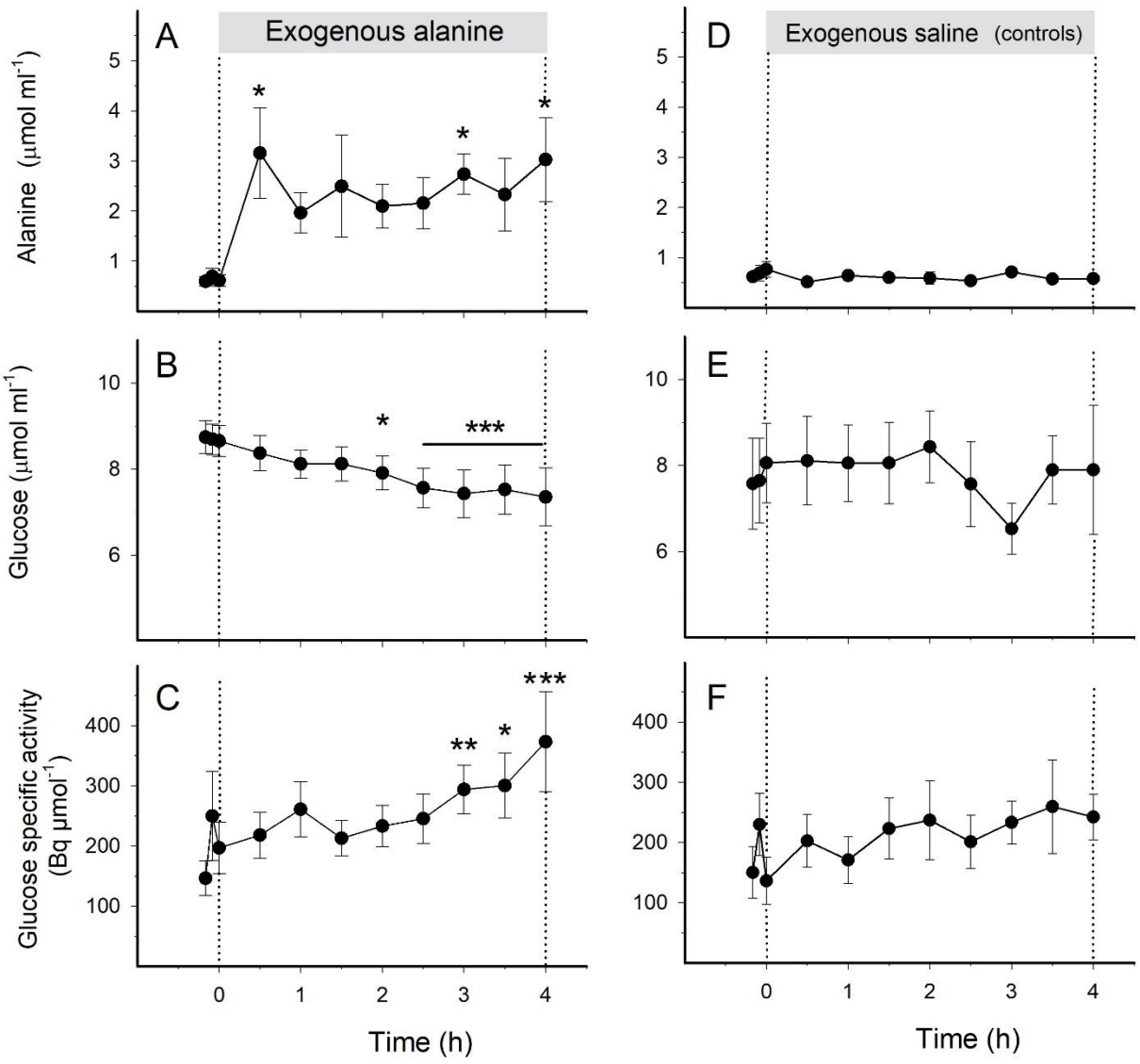

Fig. 2.1. Effects of exogenous alanine or saline administration on trout plasma metabolites and glucose specific activity during the measurement of glucose kinetics. Alanine concentration, glucose concentration, and glucose specific activity were measured for the alanine (A, B, and C respectively) and the control (D, E, and F respectively) groups. Values are means ± s.e.m. (N=6-9 for the alanine group and N=7 for the control). Means significantly different from baseline are indicated by asterisks (* P<0.05; ** P<0.01; *** P<0.001)**.** Saline had no effect on these parameters (P>0.05).

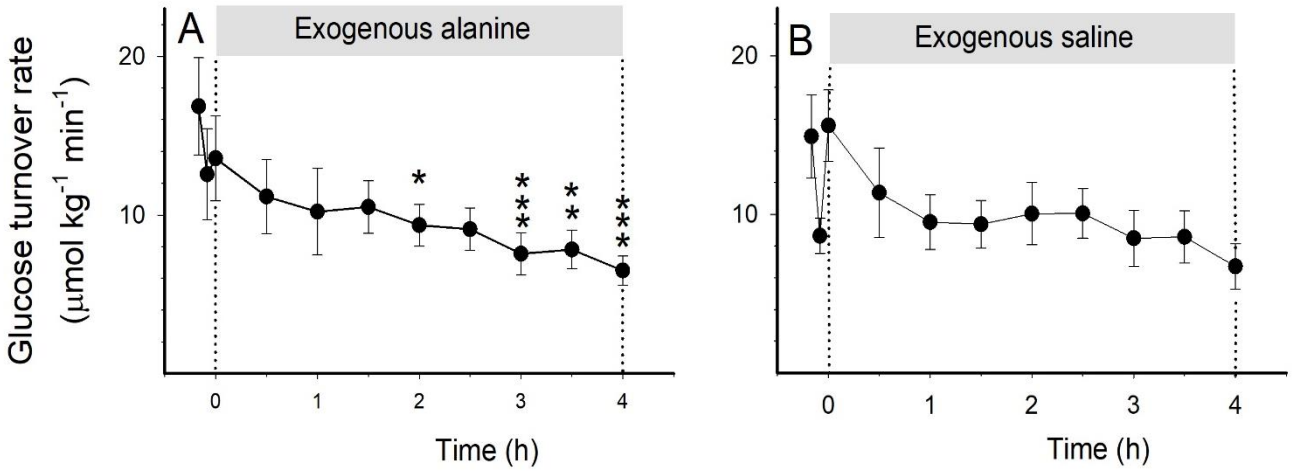

Fig. 2.2. Effects of exogenous alanine (A) or saline (B) administration on glucose turnover rate (R_t) of rainbow trout. R_t was calculated with the steady state equation of Steele (Steele, 1959). Values are means ± s.e.m. (N=8-9 for the alanine group and N=7 for the control group). Means significantly different from baseline are indicated by asterisks (* P<0.05; ** P<0.01; *** P<0.001)**.** Saline had no effect on turnover rate (P>0.05).

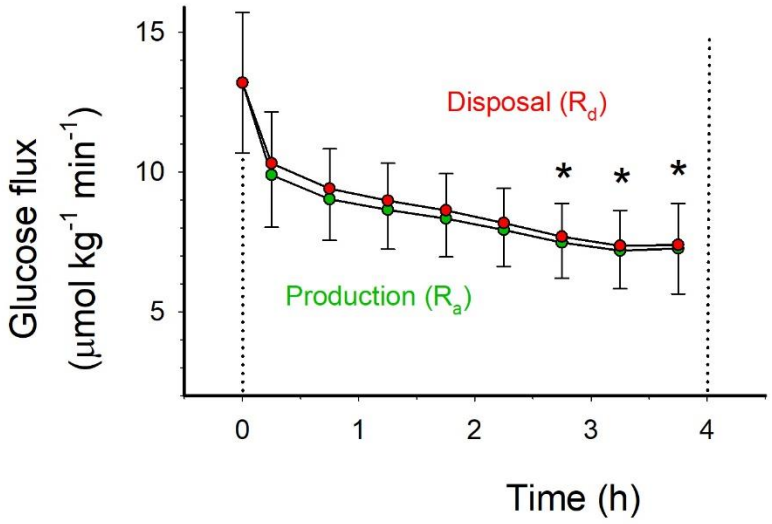

Fig. 2.3. Effects of exogenous alanine administration on the glucose kinetics of rainbow trout. The rate of glucose production (or appearance in the circulation: R_a) and rate of glucose disposal (or disappearance from the circulation: R_d) were calculated with the non steady-state equations of Steele (Steele, 1959). Values are means ± s.e.m. (N=9). Means significantly different from baseline are indicated by asterisks (* P<0.05).

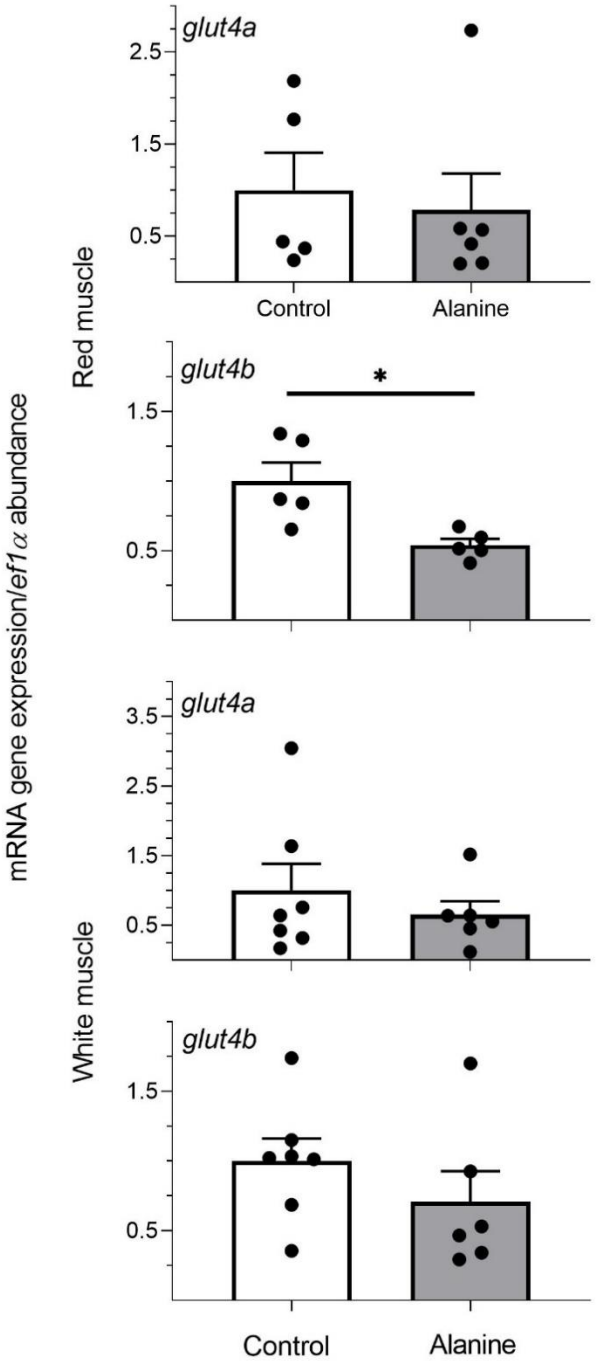

Fig. 2.4. Red and white muscle relative mRNA abundance of *glucose transporter* 4 (*glut4a* and *glut4b*) in the control and alanine-infused rainbow trout. Data were normalized to the reference gene *ef1α*. The mean + s.e.m. are represented (N=5-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$).

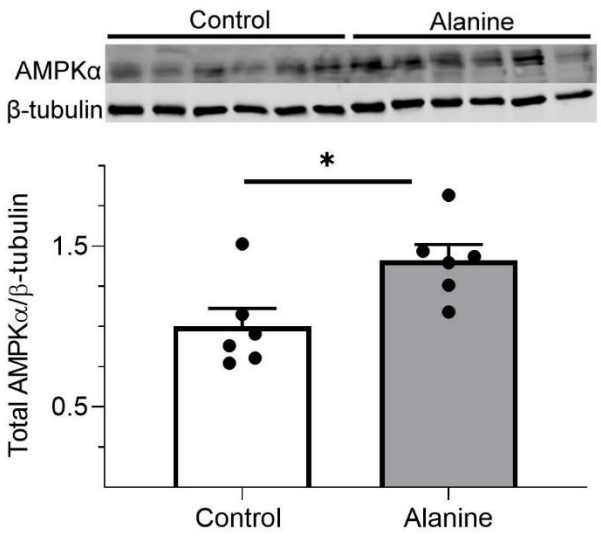

Fig. 2.5. Relative level of total AMPKα in the liver in the control and the alanine-infused groups. Data were normalized to β-tubulin and are represented as fold changes relative to the control group. The western blot is shown on top of the figure. The mean + s.e.m. are represented (N=6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$).

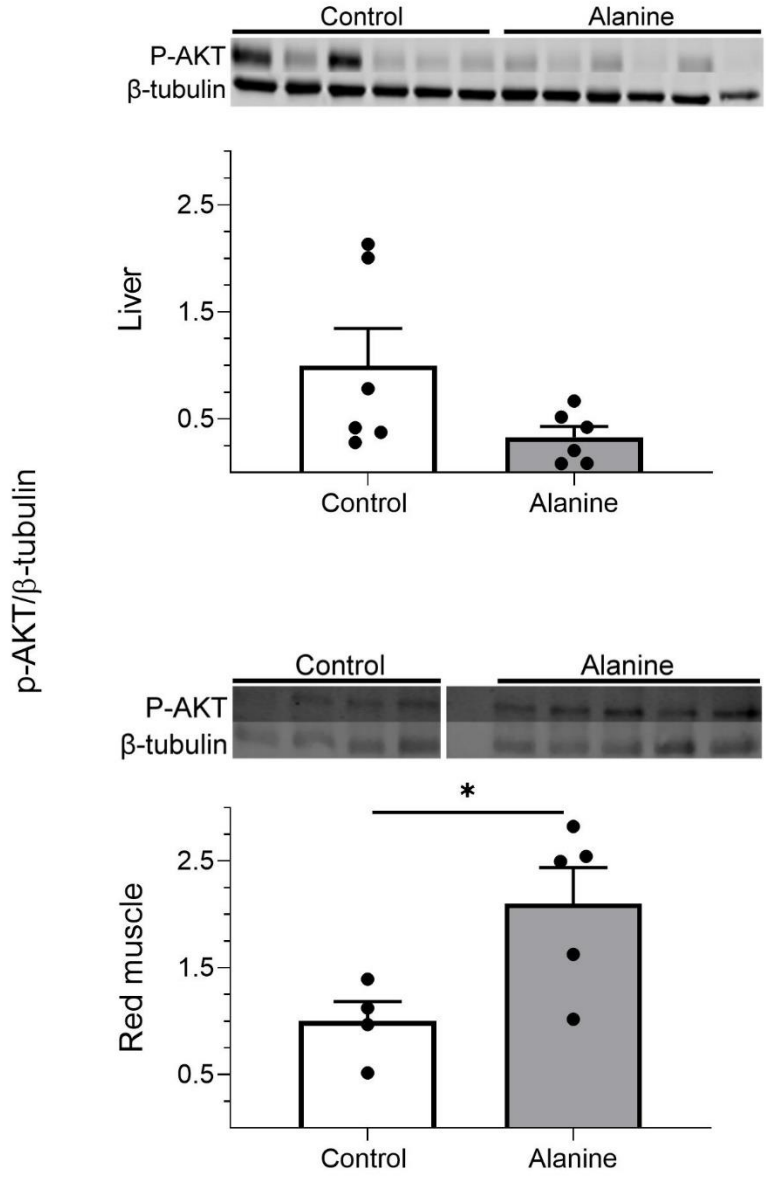

Fig. 2.6. Relative level of phosphorylated AKT (at S473) in the liver and red muscle in the control and the alanine-infused groups. Data were normalized by β-tubulin and are represented as fold changes relative to the control group. The western blot of each phosphorylated protein is shown on top of its figure. The mean + s.e.m. are represented (N=4-6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$). A white space indicates the removal of a lane (outlier).

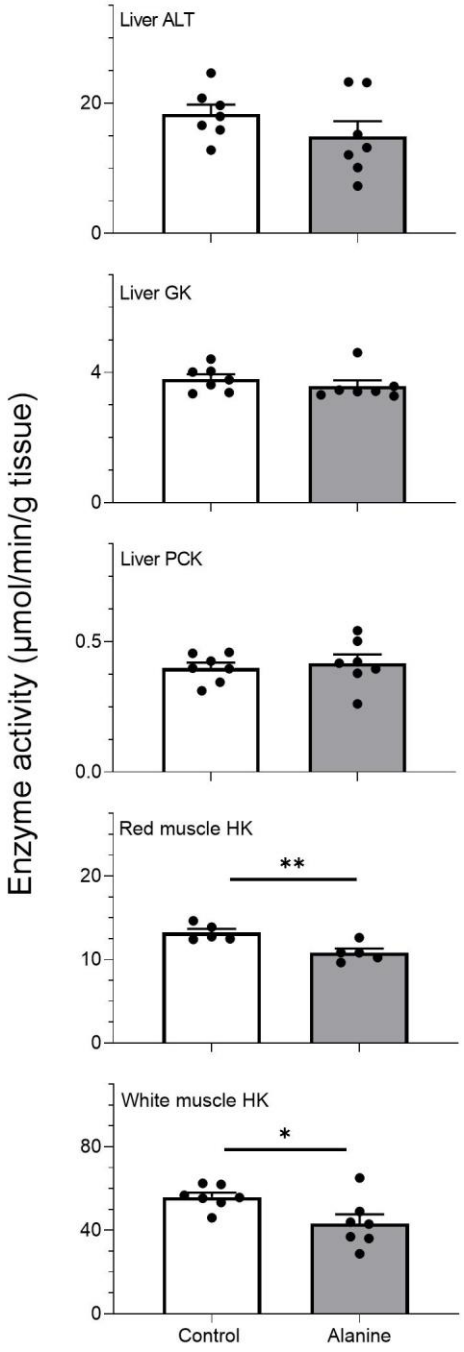

Fig. 2.7. Liver and muscle enzyme activity (µmol/min/g tissue) for alanine aminotransferase (ALT), glucokinase (GK), phosphoenolpyruvate carboxykinase (PCK) and hexokinase (HK) in the control and the alanine-infused groups. The mean + s.e.m. are represented (N=5-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Means significantly different from control are indicated by asterisks (* $P<0.05$; ** $P<0.01$).

Supplementary data

Table S2.1. Primer pair conditions used for mRNA quantification by real-time RT PCR.

mRNA	Primer sequence (5' to 3')	Annealing temperature °C	Efficiency %, R^2	Reference
pck1	F: ACAGGGTGAGGCAGATGTAGG R: CTAGTCTGTGGAGGTCTAAGGGC	55	91.9, 0.995	Marandel et al., 2015
pck2a	F: ACAATGAGATGATGTGACTGCA R: TGCTCCATCACCTACAACCT	55	90.4, 0.997	Marandel et al., 2015
pck2b	F: AGTAGGAGCAGGGACAGGAT R: CCGTTCAGCAAAGGTTAGGC	55	102.8, 0.989	Marandel et al., 2019
g6pca	F: GATGGCTTGACGTTCTCCT R: AGATCCAGGAGAGTCCTCC	55	91.9, 0.995	Marandel et al., 2015
g6pcb1a	F: GCAAGGTCCAAAGATCAGGC R: GCCAATGTGAGATGTGATGGG	59	105.9, 0.975	Marandel et al., 2015
g6pcb1b	F: GCTACAGTGCTCTCCTTCTG R: TCACCCCATAGCCCTGAAA	55	91.6, 0.997	Marandel et al., 2015
g6pcb2a	F: ATCGGACAATACACACAGAACT R: CAACTGATCTATAGCTGCTGCCT	54	91.3, 0.994	Marandel et al., 2015
g6pcb2b	F: CCTCTGCTCTTCTGACGTAG R: TGTCCATGGCTGCTCTCTAG	55	92.3, 0.985	Marandel et al., 2015
gka	F: CTGCCCACCTACGTCTGT R: GTCATGGCGTCCTCAGAGAT	54	96.3, 0.993	Marandel et al., 2015
gkb	F: TCTGTGCTAGAGACAGCCC R: CATTTTGACGCTGGACTCCT	57	90.9, 0.993	Marandel et al., 2015
hk2	F: TGAAAAGGGACATGCAGAGA R: GGCCCTAAAAGCAAGGAAA	58	92.3-96.1,0.992-0.988	Designed
glut4a	F: CATCTTTGCAGTGCTCCTTG R: CAGCTCTGTACTCTGCTTGC	56	106.1-101.1, 0.997-0.982	Liu et al., 2017
glut4b	F: TCGGCTTTGGCTTCCAATATG R: GTTTGCTGAAGGTGTTGGAG	56	101.4-106.2, 0.995-0.996	Liu et al., 2017
β-actin	F: AGAGCTACGAGCTGCCTGAC R: GTGTTGGCGTACAGGTCCTT	60	90.4, 0.996	Moltesen et al., 2016

ef1α	F: CACATCGCCTGCAAGTTT R: GAAGCTCTCCACACACATGG	58	106.6-110.1, 0.985-0.988	Designed

F and R represent forward and reverse primer sequences respectively. Primer sequences for *hk2* and ***ef1α*** was designed using Primer 3 algorithm. The efficiency and R^2 values are presented for both the red and white muscle respectively for *glut4a*, *glut4b*, *hk2* and *ef1α*. The reference genes for the liver and muscle are ***β-actin*** and ***ef1α*** respectively.

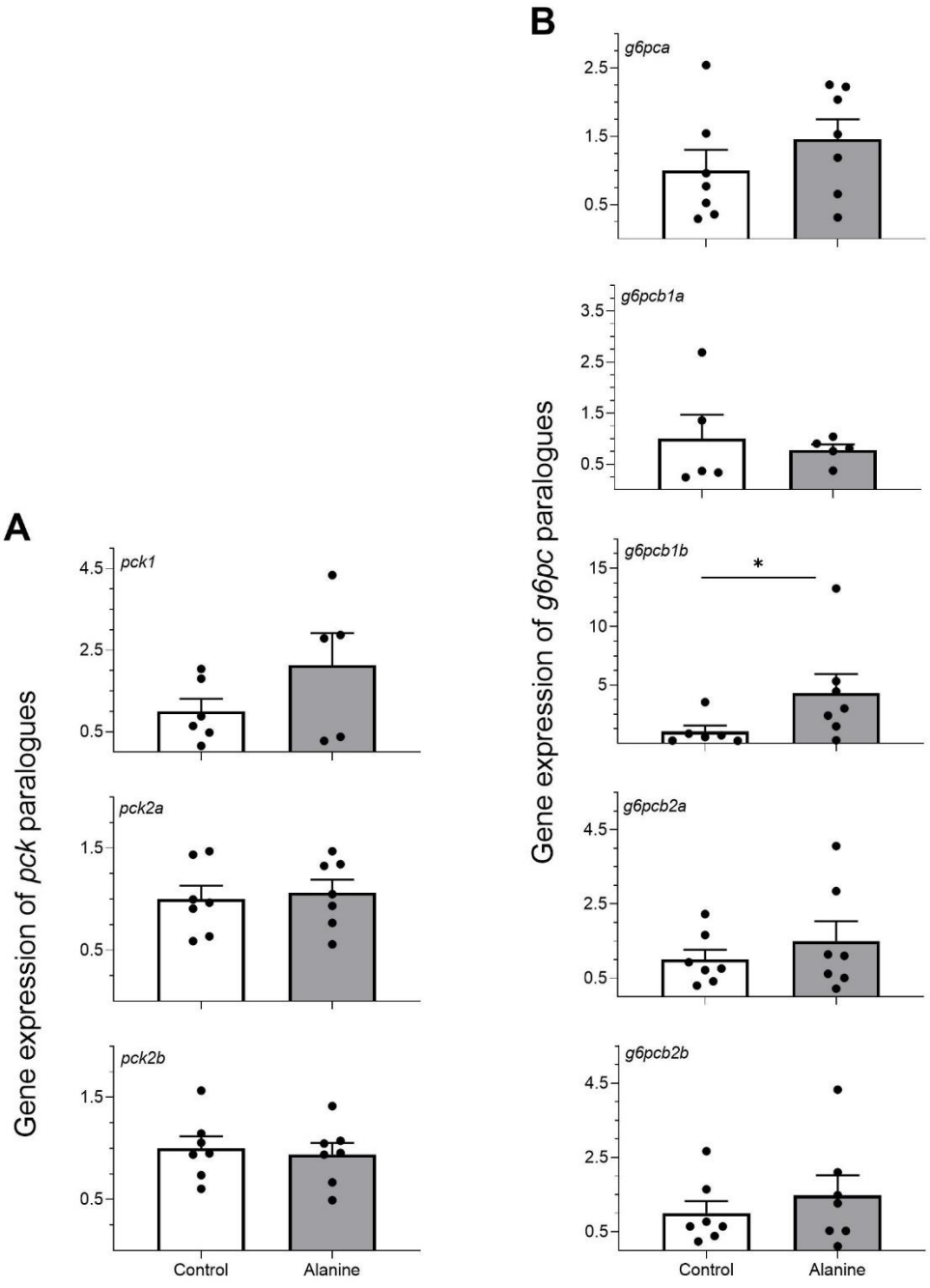

Fig. S2.1. Liver relative mRNA abundance of (A) phosphoenolpyruvate carboxykinase (*pck*) and (B) glucose-6-phosphatase (*g6pc*) paralogues in the control and alanine-infused rainbow trout. Data were normalized by the reference gene *β-actin*. The mean + s.e.m. are represented (N=5-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$).

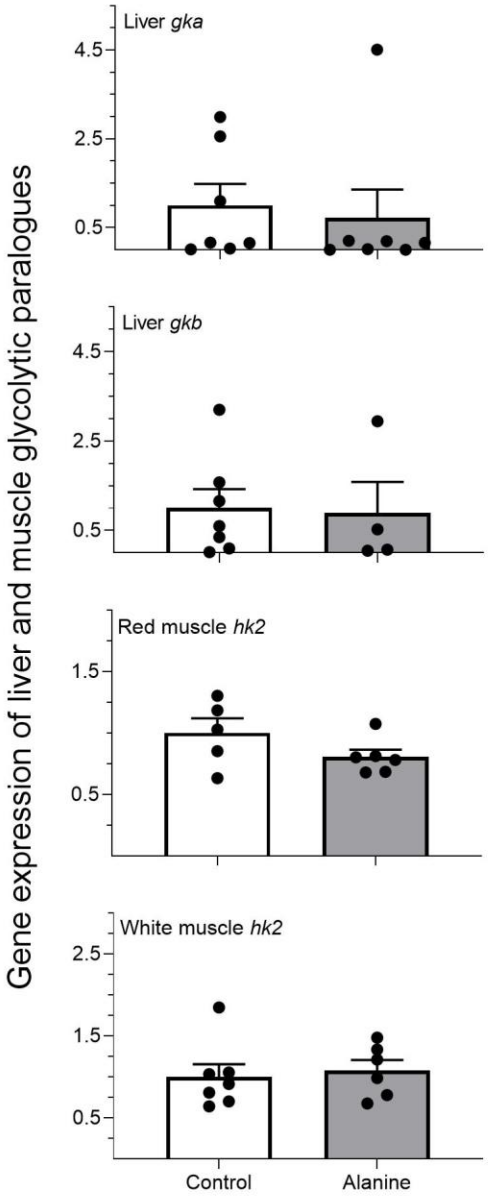

Fig. S2.2. Relative mRNA abundance of glucokinase (*gk*) in the liver and hexokinase 2 (*hk2*) in the red and white muscle in the control and alanine-infused rainbow trout. Data were normalized by the reference gene *β-actin* for the liver and *ef1α* for red and white muscle. The mean + s.e.m. are represented (N=4-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Alanine had no effect on the measured glycolytic mRNA transcript abundance ($p>0.05$).

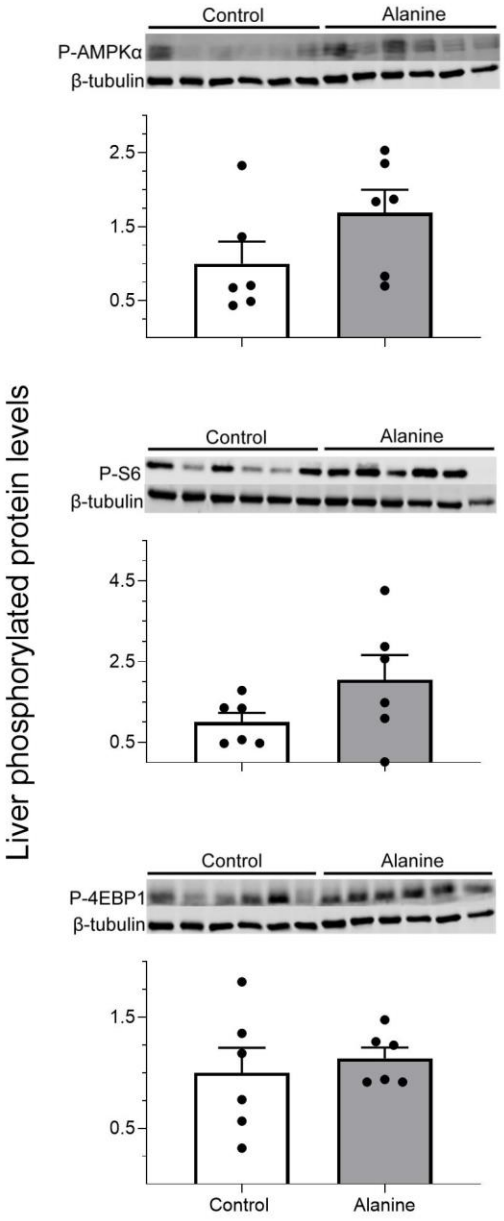

Fig. S2.3. Liver relative abundance of phosphorylated AMPKα (at T172), ribosomal protein S6 (S6; at S235/236) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1; at T37/46) in the control and the alanine-infused groups. Data were normalized by β-tubulin and are represented as fold changes relative to the control group. The western blot of each phosphorylated protein is shown on top of its figure. The mean + s.e.m. are represented (N=6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Alanine had no effect on the phosphorylated level of these proteins in the liver ($p>0.05$).

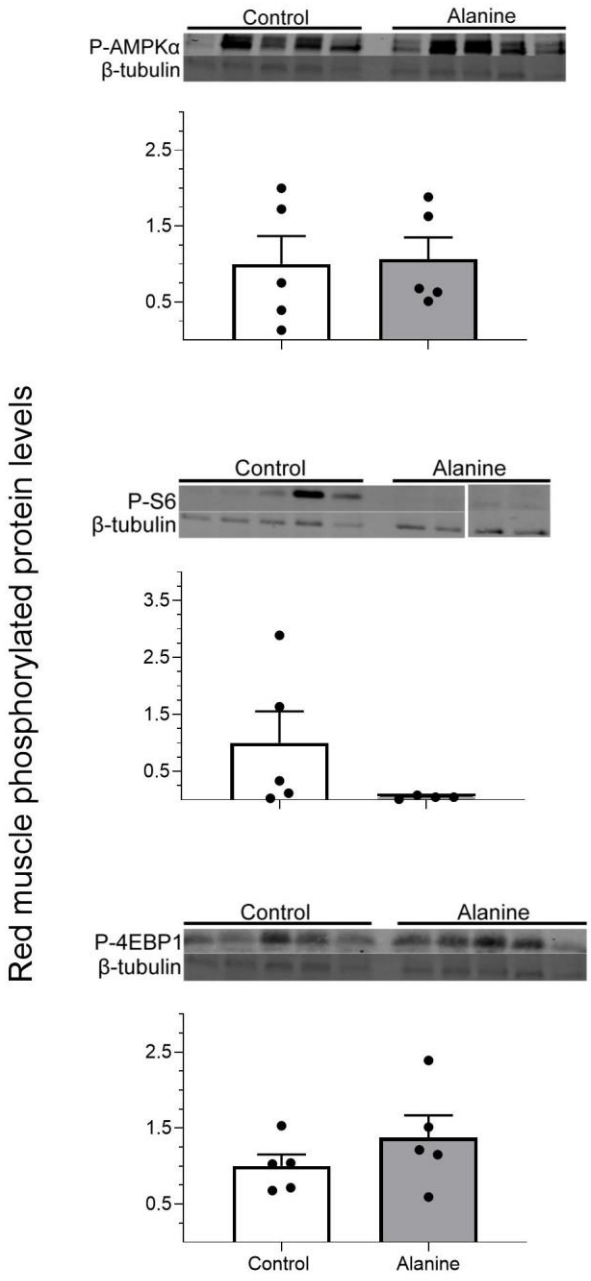

Fig. S2.4. Red muscle relative levels of phosphorylated AMPKα (at T172), S6 (at S235/236) and 4EBP1 (at T37/46) in the control and the alanine-infused groups. Data were normalized by β-tubulin and are represented as fold changes relative to the control group. The western blot of each phosphorylated protein is shown on top of its figure. The mean + s.e.m. are represented (N=4-5). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Alanine had no effect on the phosphorylated level of these proteins in the red muscle ($p>0.05$). A white space indicates the removal of a lane (outlier).

Chapter 3: General Discussion

Comparing the effects of alanine and insulin

Some amino acids are considered as better insulin secretagogues compared to carbohydrates in carnivorous fish whose diet is comparatively higher in proteins/amino acids (Panserat et al., 2013). A low-protein high-carbohydrate diet (60 days) has been shown to reduce plasma insulin levels, analyzed by a radioimmunoassay with bonito insulin, in rainbow trout (Capilla et al., 2002). Alanine was previously reported to elevate plasma insulin levels in coho salmon; however, arginine's effect was found to be the most potent in this regard (Plisetskaya et al., 1991). My results suggest that alanine stimulates the secretion of insulin which could indirectly mediate at least some of the amino acid's effects on carbohydrate metabolism. This conclusion is based on several lines of evidence from the current study when compared to insulin effects previously investigated in rainbow trout in an identical experimental setup (Forbes et al., 2019a). The comparison between alanine and insulin effects on key indices of rainbow trout glucose metabolism, including plasma glucose concentrations and fluxes, glucose transport, utilization and production as assessed at the gene expression or enzyme activity level and the activity of key proteins in the insulin signaling pathway, are detailed below (mainly based on Table 3.1). Both exogenous alanine and insulin reduced glucose concentrations and glucose fluxes, R_a and R_d, over time (see Table 3.1). In addition to Forbes and colleagues (Forbes et al., 2019a), other studies found that insulin reduced plasma glucose levels at single timepoints in rainbow trout (Plagnes-Juan et al., 2008; Polakof et al., 2010a) and brown trout (Díaz et al., 2007).

The reduction in R_d glucose after alanine infusion can be partially explained by the decrease in *glut4b* expression in red muscle and hexokinase activity in red and

white muscle. An intraperitoneal administration of insulin reduced *glut4* expression in white muscle after 6h (Polakof et al., 2010a). In the current study, the decrease in *glut4b* expression was found in red muscle after 4h of alanine infusion, while a similar trend was observed in white muscle. Indeed, correlative analysis (more details are provided in the next section) suggests that *glut4b* expression was correlated in red and white muscle. Conversely, a differential response between red and white muscle was previously reported for *glut4* gene expression in brown trout and rainbow trout (Capilla et al., 2002) or protein content in brown trout (Díaz et al., 2007). The paradoxical reduction in *glut4* expression in muscle under increased insulin levels in trout was previously suggested to be due to a counterregulatory response (Polakof et al., 2010a). The white muscle was suggested to be involved in this response. Specifically, after treatment with insulin, the white muscle triggered a large glucose uptake, due to its mass, and increased glycogen levels which largely explained the acute hypoglycemia and was suggested to consequently contribute to the decrease in glucose uptake (Polakof et al., 2010a). Unfortunately, neither alanine's effect on glycogen level nor the expression and activity of key genes and proteins involved in glycogen metabolism (synthesis and degradation) were assessed in the current study. Considering the results of the current study, I suggest that the decrease in glucose turnover, or the rate of glucose production and utilization, and glycemia after 4h of alanine infusion, which was probably mediated by insulin, contributed to the decreased expression of *glut4b* in red muscle. Other studies with insulin treatment conducted over longer periods of time (24h or 30h) reported an increase in *glut4* expression in white muscle (Polakof et al., 2010b) or only in red muscle (Capilla et al., 2002). When insulin was administered for days (4-

5), either an increase (Polakof et al., 2010a) or no significant change (Polakof et al., 2010b) were observed in the transcript abundance of *glut4* in white muscle. In the above-mentioned studies, the different *glut4* isoforms were not investigated because rainbow trout genome was only fully sequenced in 2014 (Berthelot et al., 2014). However, it is important to note that the assessment of *glut4* expression is not sufficient evidence for changes in glucose transport because the most critical step in the regulation of GLUT4 is its translocation to the plasma membrane (Yamamoto et al., 2016). Therefore, future studies are warranted to investigate the effect of alanine or insulin on GLUT4 translocation in rainbow trout using protocols previously validated in mammals (Yamamoto et al., 2016). Hexokinase relative transcript abundance and activity were measured as an index of glycolytic activity in muscle. HK activity, but not transcript levels, decreased after 4h of alanine administration in both red and white muscle. When considering the cellular fate of glucose, the reduction in hexokinase activity is consistent with the decrease in *glut4* expression, suggesting a reduced capacity to import and metabolize glucose. After 6h of insulin administration, a decrease in *hk* expression was observed in trout (Polakof et al., 2010a). Unfortunately, HK activity was not measured in the latter study. The effect of insulin on hexokinase seems to be time-dependent because when this hormone was administered for longer periods (30h, 4 or 5d) through mini-osmotic pumps, it had no effect on *hk* expression (Polakof et al., 2010a; Polakof et al., 2010b) or it caused a slight non-significant increase in *hk* expression and activity (Polakof et al., 2011b).

In the liver, glucokinase activity was measured as an index of glycolysis. Gene expression and activity of GK were not modified by alanine after 4h. Conversely, the

expression of *gk* was reduced 6h, 4 or 5 days after insulin treatment in trout (Polakof et al., 2010a; Polakof et al., 2010b). However, the response of GK activity and expression was found to be uncorrelated with plasma insulin levels in rainbow trout (Capilla et al., 2003). This may be linked to the fact that hepatic GK in rainbow trout is considered to be more responsive to glucose than insulin, in contrast to the response found in mammals and birds (Panserat et al., 2014). Furthermore, GK activity was confirmed to be a robust indicator of dietary carbohydrate intake (Marandel et al., 2015). Gene expression and activity of hepatic GK were previously shown to increase in proportion to starch content in the diet (Capilla et al., 2003) or after refeeding (Soengas et al., 2006) in rainbow trout. However, in the current study, the decrease in glucose levels were probably not strong enough to elicit changes in GK activity or expression.

With regards to the gluconeogenic pathway, the activity of PCK, a rate-limiting enzyme, was assessed. According to my results, PCK activity did not change after 4h of alanine treatment. In rainbow trout fed a high protein diet, insulin reduced PCK activity (Cowey et al., 1977a). One possible explanation for this discrepancy is the different nature of experimental design of the current study and Cowey's experiments because they did a dietary treatment over 6 weeks in juvenile rainbow trout. However, a dietary alanine treatment (over 9 weeks) was found to reduce the activity of key gluconeogenic enzymes, including PCK, in juvenile trout (Kirchner et al., 2003a). Therefore, a dietary treatment with alanine or insulin over longer periods of time similarly reduced PCK activity in juvenile rainbow trout and this suggests that the timeframe of alanine treatment in the current study was insufficient to elicit a response at the activity level.

When considering key indicators of the intracellular insulin signaling pathway, an increase in red muscle p-AKT but no change in p-S6 were observed after 4h of alanine treatment. Due to technical issues, the white muscle proteins were not analyzed. Both p-AKT and p-S6 increased in white muscle after insulin infusion in trout (Forbes et al., 2019a). In the liver, however, no change was observed in the levels of both proteins after treatment with alanine (current study) or insulin (Forbes et al., 2019a). A differential response to insulin treatment between the liver and muscle was previously reported in other studies. Total AKT increased only in muscle but not in liver after 6h of insulin administration by an intraperitoneal injection (Polakof et al., 2010a). Moreover, p-AKT increased in white muscle and adipose tissue but not in liver 30h or 4-5d after chronic insulin treatment (Polakof et al., 2010b; Polakof et al., 2011b). Interestingly, correlative analyses (more details are provided in the next section) provided additional support regarding potential differential responsiveness seen between the two tissues. In the liver, p-AKT was correlated with glucose levels while p-S6 was correlated with alanine (Fig. 3.6). However, the opposite is seen in muscle (Fig. 3.7), supporting the different response to exogenous alanine seen in liver and muscle. These pieces of evidence suggest that the liver has a different response/sensibility to alanine and/or insulin compared to muscle. It is conceivable that the liver exhibits different kinetics and feedback mechanisms compared to muscle, and that due to the experimental limitations of terminal tissue sampling at individual timepoints (after 4h), potential kinetic information was lost. It is important to discuss the effects of alanine and insulin on AMPK in trout because insulin is known to inhibit AMPK activity in mammals through direct phosphorylation mediated by AKT (Jeon, 2016; Kjøbsted et al., 2018). Total

AMPK levels increased in liver after alanine treatment. However, p-AMPK did not change after 4h of alanine infusion in liver and muscle, which is similar to observations 3h following a single bolus, intraperitoneal insulin administration (Jin et al., 2014). This suggests that AMPK activity did not change in response to insulin or alanine, which could potentially increase the secretion of this hormone. It is possible that insulin levels were not sufficient to induce changes in AMPK activity, especially in the liver where p-AKT did not change significantly and its levels showed a decreased trend after 4h of alanine infusion, suggesting a lower response in liver to the potential increase in insulin secretion mediated by alanine.

Correlation analysis of key indices in the gluconeogenic and glycolytic pathways

Correlative analyses (heatmaps) were performed to explore relationships between the measured endpoints. This type of analysis is exploratory and does not provide a cause and effect relationship. However, correlative analysis is useful to generate hypotheses which can be tested functionally in future studies (Kostyniuk et al., 2019b). Given that the rate of production and utilization of glucose were quantified *in vivo*, it was relevant to separate the variables measured for the molecular part by pathways, specifically gluconeogenesis and glycolysis, for correlative analysis. Both R_a and R_d glucose as well as plasma glucose levels decreased when exogenous alanine was administered. Therefore, the correlation, or lack of it, between the quantified gluconeogenic or glycolytic enzymes, at the activity and expression levels, was investigated. For instance, it was of interest to obtain insights as to whether plasma alanine is correlated with plasma glucose or the measured glycolytic and gluconeogenic enzymes, whether the activity and expression of a given enzyme are correlated and

whether the duplicated copies (paralogues) of a given gene are correlated. In Fig. 3.1 (gluconeogenesis), alanine levels were correlated with different gluconeogenic genes, at the expression level, such as *g6pca*, *pck1* and *g6pcb1b*. This suggests that alanine as a gluconeogenic amino acid may regulate gluconeogenesis through these genes. It is also clear from the heatmap in Fig. 3.1 that PCK activity and gene expression were not well correlated. It is possible that the regulation of the enzyme activity by post-translational modification, phosphorylation for instance, is different from the dynamic in gene expression. At the expression level, the different *pck* paralogues were not well correlated. A differential regulation of *pck2a* and *pck2b* expression was previously reported in rainbow trout under different conditions known to modify gluconeogenesis suggesting functional diversification between the two paralogues (Marandel et al., 2019). On the other hand, *g6pcb2a* and *g6pcb2b* were highly correlated. The correlation between *g6pcb2a* and *g6pcb2b* was previously reported in rainbow trout and a high identity percentage in the coding region of these paralogues was found to be associated with their expression patterns (Marandel et al., 2015). These duplicated genes were suggested to be retained after the salmonid-specific whole genome duplication event indicating a lesser evolutionary distance compared to other *g6pc* paralogues that evolved prior in teleost (Marandel et al., 2015). Additionally, *g6pcb2a* and *g6pcb2b* were correlated with glucose levels and *pck2b* expression. The expression of *g6pcb2* paralogues increased in trout fed a high carbohydrate diet (Marandel et al., 2015). Furthermore, the expression of *pck2b* was upregulated in trout fed a high-carbohydrate diet that induced hyperglycemia (Marandel et al., 2019). Therefore, findings from other studies, as discussed above, support correlative evidence in the current study and

suggest a potential contribution of the expression of *g6pcb2a*, *g6pcb2b* and *pck2b* to the atypical regulation of gluconeogenesis when carbohydrates are available in rainbow trout. In Fig. 3.2 (glycolysis), glucokinase paralogues *gka* and *gkb* were correlated together and with hexokinase activity in white muscle and glucose levels, but they were less correlated with glucokinase activity. The correlation between *gk* expression and glucose levels is reasonable because the expression and activity of GK were previously found to increase significantly in rainbow trout fed a high-carbohydrate diet (Marandel et al., 2015). The expression of *glut4a* is correlated in red and white muscle and with *hk2* mRNA levels in white muscle. Additionally, Fig. 3.2 shows that *glut4b* is well correlated with *hk2* expression in red muscle and both are correlated with *glut4b* in white muscle. Interestingly, there is correlation between the clusters of *glut4a* and *glut4b* indicating a correlation between the two paralogues. Therefore, these correlative results suggest that the expression of key genes in glucose transport and utilization is coordinated in muscle.

Significance of the study in the context of aquaculture

Salmonids represent one of the most cultured species around the world. This family received significant interest because many of its species are important in aquaculture and studies on their nutritional needs are available as previously reviewed (Panserat et al., 2013). One of the important freshwater aquaculture salmonid species is rainbow trout. The high dietary protein requirements of carnivorous fish are supplied by aquafeeds which are economically and environmentally expensive as they are based on marine resources and are mainly made of fish meal and oil (Panserat et al., 2013). Therefore, efforts focused on investigating the replacement of fish meal and oil by

alternatives that are more sustainable and less costly such as plant-based proteins, carbohydrates and vegetable oil to minimize exploiting threatened marine resources. Carbohydrates and especially starch, are largely available and less costly than fish meal. Even if carbohydrates are digested and absorbed, after treatments to facilitate these processes, they are poorly metabolized in trout. In fact, digestible dietary carbohydrates are limited to 20% of the diet due to decreased growth or fatty liver associated with the observed hyperglycemia when higher amount of carbohydrates are consumed (Panserat et al., 2013). Plant proteins are lacking in essential amino acids including lysine and methionine (Dai, 2015). Proteins/amino acids have crucial roles in fish including tissue growth, energy production and synthesis of other compounds as previously explained in the introduction of this thesis. Partial replacement of fish meal and oil by plant proteins and vegetable oils was achieved in fish diets, but there is still no complete replacement due to growth issues (Panserat et al., 2019). The replacement of fish meal with alternatives needs a thorough knowledge of nutritional needs and intermediary metabolism. New feeding strategies, such as nutritional programming, were introduced and tested on fish to improve their nutrition and try reducing the dependance of aquaculture on aquafeeds (Panserat et al., 2019). One strategy in nutritional programing is the exposure to a dietary stimulus in early life stages that can affect feeding habits and potentially improve fish nutrition. For instance, the intake of carbohydrate-rich or plant-based diets at first feeding was tested in juvenile trout (Panserat et al., 2019).

In the context of aquaculture, it is important to answer the following question: does this thesis help in providing new ideas regarding the formulation of

environmentally sustainable feeds? The current study only represents a starting step toward a better understanding of the effect of individual amino acids on glucose metabolism by integrating glucose fluxes at the whole animal level and specific molecular indices measured in tissues. The current results suggest that glucose utilization was reduced when more alanine was made available. Because this study was not done in a physiological situation, a dietary treatment containing an excess of alanine alone or combined with high amounts of carbohydrates is an essential future step to better answer the question and investigate alanine's effects on glucose metabolism in the presence of dietary carbohydrates. The partial replacement of dietary protein by alanine in the diet (over 9 weeks) decreased key gluconeogenic enzyme activities, contrary to predictions, with no significant effects on postprandial glycemia or growth rates in juvenile rainbow trout (Kirchner et al., 2003a). There is no such study on adult rainbow trout to our knowledge.

Study limitations and future directions

First, there was an observed variability within individuals from both the alanine and control groups, especially for fish used for the molecular part of the study. Many of the assessed parameters were similar in both groups and individual variability could partially explain that. The variability among individuals could be seen in most of the heatmaps (Fig. 3.1, Fig. 3.6 and Fig. 3.7). A sample size of 4 to 7 fish was used to assess molecular indices as in previous studies by others (Polakof et al., 2010a; Polakof et al., 2011b; Soengas et al., 2006). Even though natural variability exists between fish, it would have been better to increase the sample size to improve the chances to detect biologically meaningful differences. However, it is important to

mention that double dorsal aorta catherization in trout is a challenging surgery that takes much time and effort. Therefore, adding only 2-3 fish for each group could have been feasible, but would have limited the time dedicated to molecular measurements. Second, the parameters for molecular analysis were assessed at a specific time point (after 4h of infusion). Therefore, measuring more time points between 0h and 4h could have provided more information to get a better picture of alanine's effects over time. Third, other key enzymes in glucose metabolism could have been measured at the activity level such as the last enzyme in gluconeogenesis, glucose-6-phosphatase, and glycolysis, pyruvate kinase, to get more insights into the effects of alanine on these pathways of interest. Fourth, even though insulin levels cannot be currently measured precisely in fish due to the lack of specific antibodies (Moon, 2001), glucagon could have been measured. Recently, when insulin was infused in rainbow trout, an increase in glucagon levels, a counterregulatory hormone, was reported (Forbes et al., 2019a). In rainbow trout, plasma concentrations of glucagon and glucagon-like peptide 1 were elevated in addition to the increase in insulin levels after arginine intraperitoneal injection (Mommsen et al., 2001). Measuring glucagon levels could have provided additional evidence for a potential counterregulatory response in the current study as previous findings in mammals reported an increase in glucagon levels after intravenous alanine administration in newborn infants (Sann et al., 1978) or alanine infusion in dogs (Müller et al., 1971). Glucagon levels were not measured due to the limited volume of blood that can be drawn as several samples were taken during the 4h period. Fifth, the normalization of p-AKT by β-tubulin (Fig. 2.6) was different in terms of significance compared to normalization by total AKT (Fig. 3.5) protein levels in red muscle, but the

trend was similar. However, normalization by β-tubulin (Fig. 2.6 and Fig. S2.3), total protein of interest (Fig. 3.3) and total proteins (Fig. 3.4) were similar in terms of significance for p-AMPK, p-AKT, p-S6 and p-4EBP1 protein levels measured in liver. Lastly, the effects of alanine were assessed in liver and muscle only. Even though the liver plays a principle role in glucose metabolism (Polakof et al., 2012) and the muscle is relatively the largest tissue in size, about 55%, in rainbow trout (Kaushik and Seiliez, 2010), it would have been informative to study additional tissues such as the brain, because it plays a key role in glucose metabolism, or gills, as the potential importance of alanine in this tissue was previously discussed (Mommsen, 1984).

There are several directions for future studies and some of them are discussed here. The current results suggest that insulin indirectly mediates alanine's effects. Therefore, it would be interesting to inhibit insulin receptor signaling using an antagonist, such as S961 that has been validated in mammals (Sharma and Kumar, 2018; Vikram and Jena, 2010), to investigate whether alanine mediates its effects via insulin or other unknown pathways. Key indices in glycogen metabolism (synthesis and degradation) could be measured in the future to understand whether they are affected by alanine as the current results indirectly suggest. It appears that alanine may cause an increase in glycogen synthesis and a decrease in its degradation. In mammals, alanine affected ammonia metabolism and was suggested to promote the urea cycle (Adachi et al., 2018). Therefore, future studies can try modifying alanine levels in a more natural way (dietary treatment) and measuring its effects on nitrogen metabolism in addition to glucose metabolism. Additionally, alanine manipulations could be done on larval trout in future studies because nitrogen metabolism and excretion is different in

early life stages compared to juvenile and adult fishes (Zimmer et al., 2017). Many interesting studies could also be performed in the context of exercise because alanine levels were reported to increase after swimming in rainbow trout (Milligan, 1997). AMPK expression and activity have also been shown to increase in response to sustained exercise in trout muscle (Magnoni et al., 2014; Morash et al., 2014). Therefore, it would be interesting to study the effects of sustained swimming on alanine metabolism and AMPK to detect potential regulatory links between signaling and protein utilization.

Conclusions

This study is the first to characterize how exogenous alanine affects glucose kinetics in rainbow trout and it provides important insights on the potential mechanisms responsible for the observed changes in glycemia and glucose fluxes. It shows that alanine reduces circulating glucose levels as well as the rates of hepatic glucose production and peripheral glucose utilization. These findings suggest that alanine mediates at least some of its effects by stimulating insulin secretion. When comparing the effects of alanine and insulin on glucose metabolism of rainbow trout, many similarities were found (see Table 3.1). At the whole-organism level, plasma glucose levels and glucose fluxes, R_a and R_d, decreased after alanine or insulin infusion. At the tissue level, *glut4* expression decreased in red muscle in the current study and in the white muscle when insulin was administered. HK activity decreased in both red and white muscle after alanine administration. These reductions in key indices of glucose transport and utilization may have been caused by the reduction in glucose fluxes and hypoglycemia mediated by higher insulin levels. Glucagon may also have been involved because insulin administration was found to increase glucagon levels in rainbow trout

(Forbes et al., 2019a). Furthermore, p-AKT increased in red muscle in the current study and in white muscle after insulin infusion. The effects on liver p-AKT, p-S6 and p-AMPK were similar after treatment with alanine or insulin. It is clear that investigating whether alanine acts as an insulin secretagogue in rainbow trout is an important avenue for future research.

Table 3.1. Comparison between the effects of alanine and insulin on different glucose metabolism related parameters.

Parameters	Alanine[a]	Insulin[b]
Plasma glucose	↓	↓
R_a glucose	↓	↓
R_d glucose	↓	↓
Red muscle *glut4a*	__	ND
Red muscle *glut4b*	↓	ND
White muscle *glut4a*/*glut4b*	__	↓ (Polakof et al., 2010a)
Red muscle HK activity	↓	ND
White muscle HK activity	↓	ND
Red muscle p-AKT	↑	ND
Red muscle p-S6	__	ND
White muscle p-AKT	ND	↑
White muscle p-S6	ND	↑
Liver p-AKT	__	__
Liver P-S6	__	__
Liver and muscle p-AMPKα	__	__ (Jin et al., 2014)
Liver GK activity	__	ND
Liver PCK activity	__	↓ (Cowey et al., 1977a)

[a] The current study

[b] The effects of insulin on the listed parameters are mainly based on (Forbes et al., 2019a) or other studies (directly cited in the table).

ND means not determined/measured. The symbols ↑, ↓ and __ indicate an increase, a decrease or no effect, respectively.

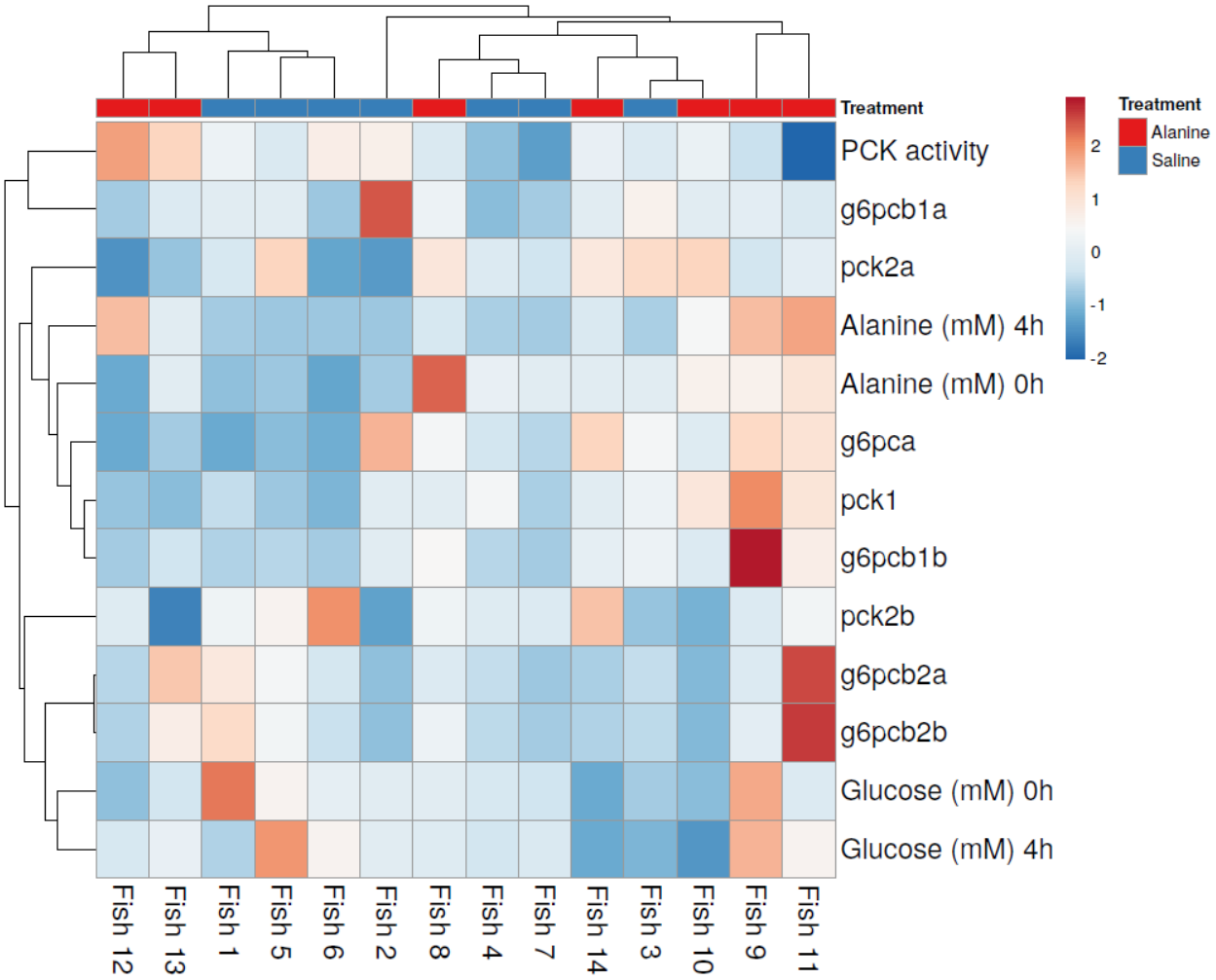

Fig. 3.1. Heatmap correlative analysis of the measured gluconeogenic indices in the liver. Fish designated for the molecular work were used for correlative analysis done by ClustVis. To generate the heatmap, standard conditions were used in ClustVis (Metsalu and Vilo, 2015). The correlation or lack of correlation is depicted in the heatmap between alanine, glucose, the activity and expression of phosphoenolpyruvate carboxykinase (PCK, *pck1*, *pck2a* and *pck2b* respectively) and the expression of glucose-6-phoshatase paralogues (*g6pcs*) in the control and alanine group.

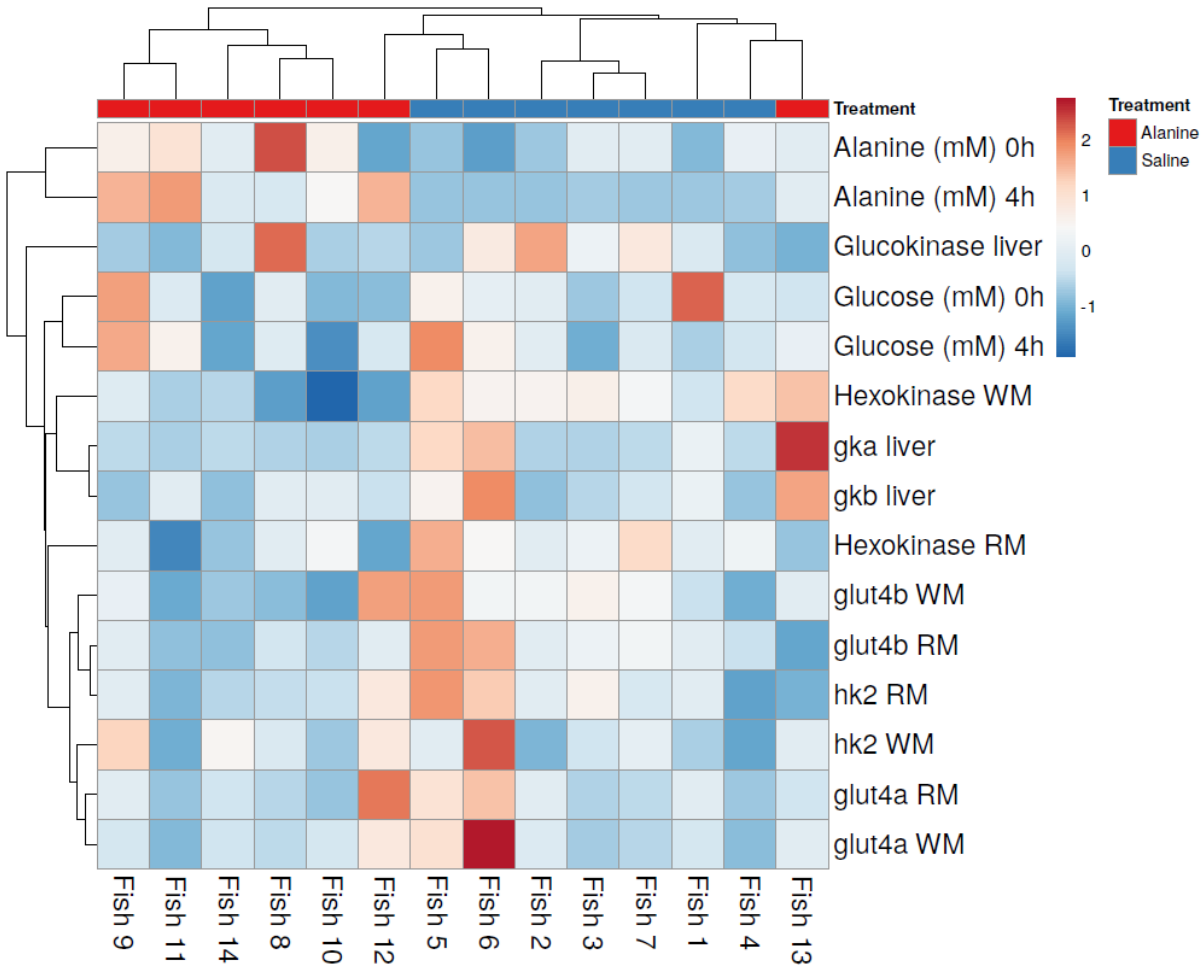

Fig. 3.2. Heatmap correlative analysis of the measured glycolytic indices in the liver, red and white muscle. Fish designated for the molecular work were used for correlative analysis done by ClustVis. To generate the heatmap, standard conditions were used in ClustVis (Metsalu and Vilo, 2015). The correlation or lack of correlation is depicted in the heatmap between alanine, glucose, the activity and expression of glucokinase (GK and *gka* and *gkb* respectively) in liver, glucose transporter 4 paralogue a and b (*glut4*), the activity and expression of hexokinase (HK and *hk2* respectively) in red and white muscle (RM and WM respectively) in the control and alanine group.

Appendix

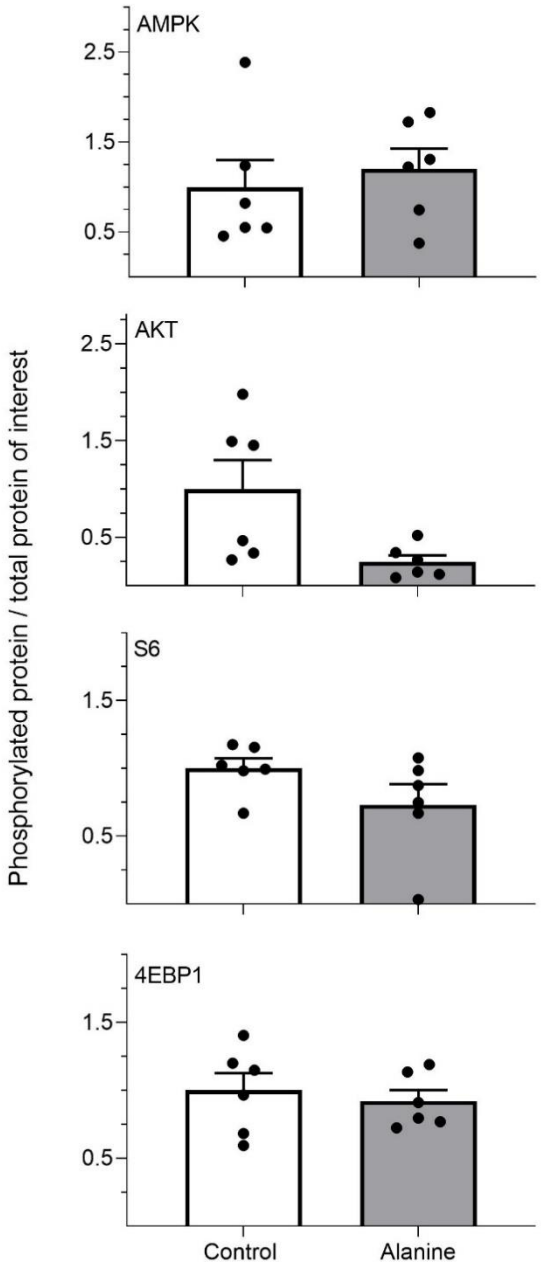

Fig. 3.3. Liver relative abundance of phosphorylated AMPK (at T172), AKT (S473), S6 (S235/236) and 4EBP1 (T37/46) in the control and the alanine-infused groups. Data were normalized by the total protein of interest and are represented as fold changes relative to the control group. The mean + s.e.m. are represented (N=6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. The p-value equals 0.511, 0.052, 0.143 and 0.612 for AMPK, AKT, S6 and 4EBP1, respectively.

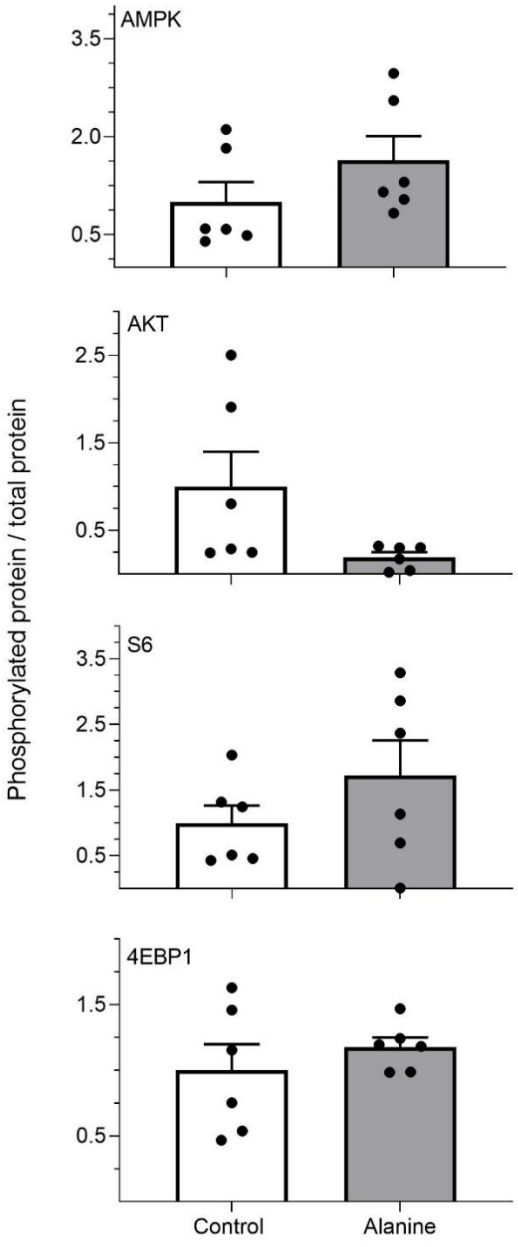

Fig. 3.4. Liver relative abundance of phosphorylated AMPK (at T172), AKT (S473), S6 (S235/236) and 4EBP1 (T37/46) in the control and the alanine-infused groups. Data were normalized by total proteins and are represented as fold changes relative to the control group. The mean + s.e.m. are represented (N=6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. The p-value equals 0.121, 0.099, 0.250 and 0.435 for AMPK, AKT, S6 and 4EBP1, respectively.

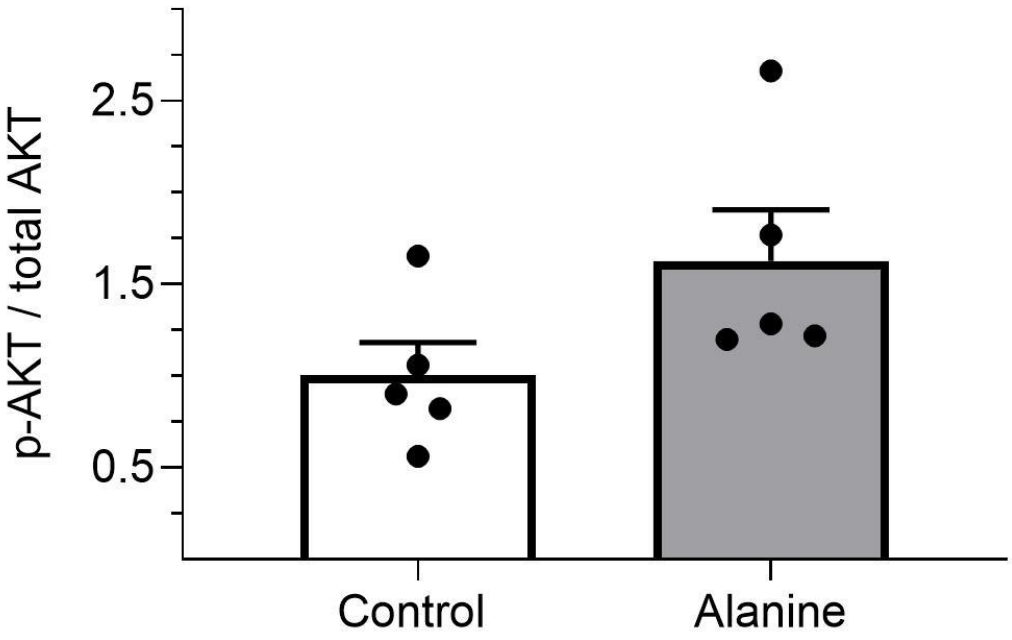

Fig. 3.5. Relative level of phosphorylated AKT (S473) in red muscle in the control and the alanine-infused groups. Data were normalized by total AKT and are represented as fold changes relative to the control group. The mean + s.e.m. are represented (N=5). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. The p-value is 0.097.

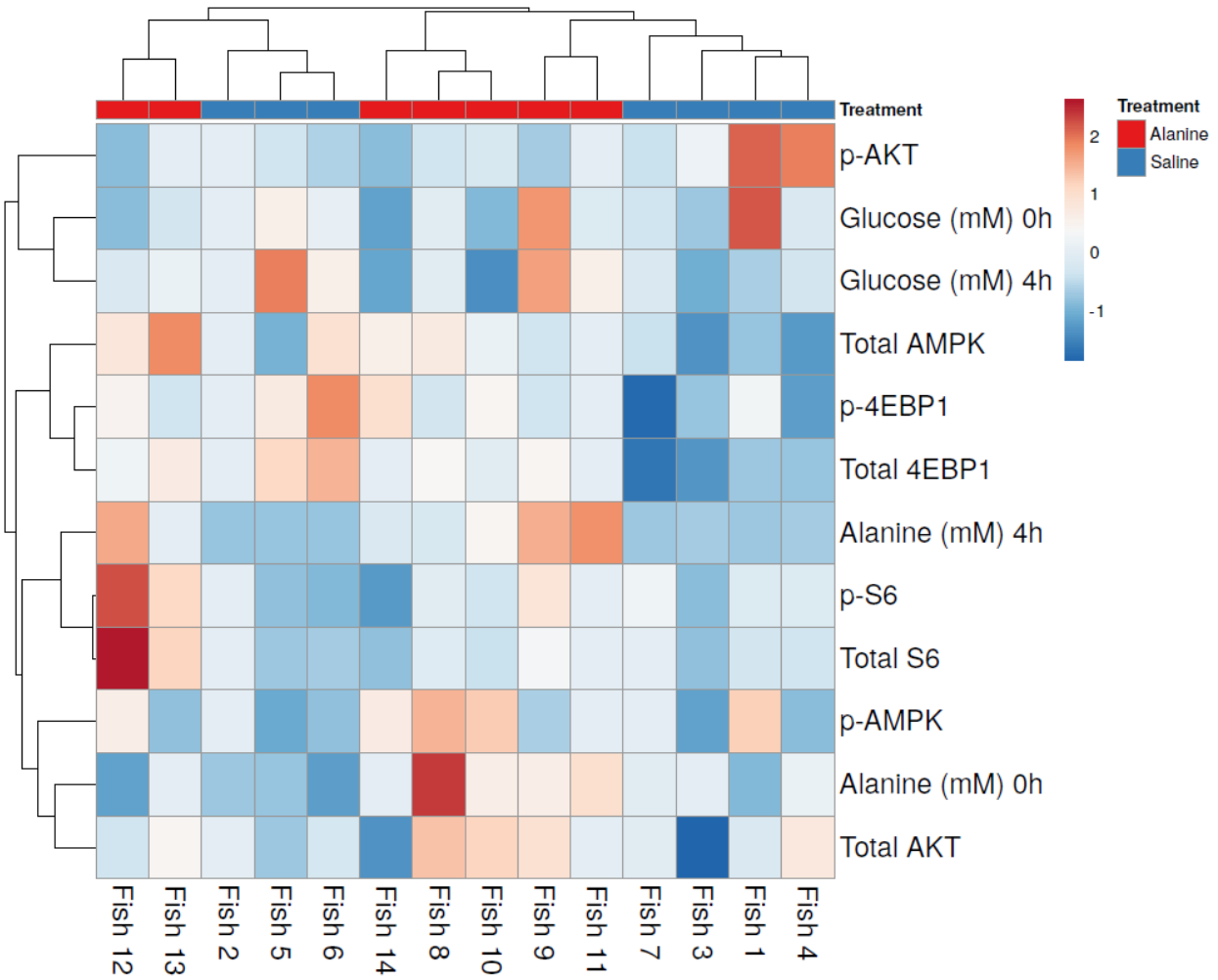

Fig. 3.6. Heatmap correlative analysis of the measured signaling indices in liver. Fish designated for the molecular work were used for correlative analysis done by ClustVis. To generate the heatmap, standard conditions were used in ClustVis (Metsalu and Vilo, 2015). The correlation or lack of correlation is depicted in the heatmap between alanine, glucose, the total and phosphorylated form of AMPK, AKT, S6, 4EBP1 in the control and alanine group.

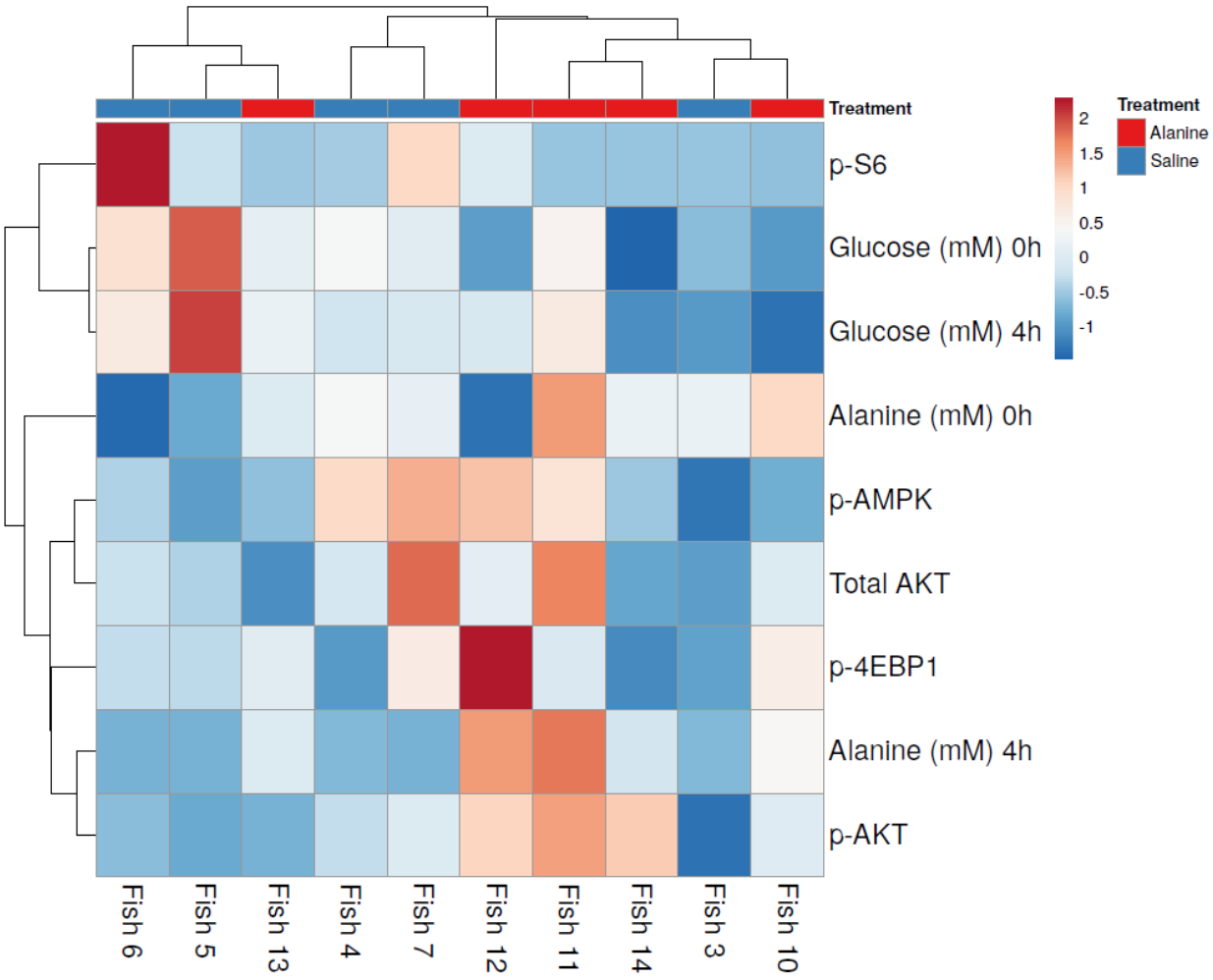

Fig. 3.7. Heatmap correlative analysis of the measured signaling indices in red muscle. Fish designated for the molecular work were used for correlative analysis done by ClustVis. To generate the heatmap, standard conditions were used in ClustVis (Metsalu and Vilo, 2015). The corre(Forbes et al., 2019a)lation or lack of correlation is depicted in the heatmap between alanine, glucose, phosphorylated form of AMPK, AKT, S6, 4EBP1 and the total from of AKT in the control and alanine group.

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