Biological availability of different commercially available histidine products in feed for Atlantic salmon smolt

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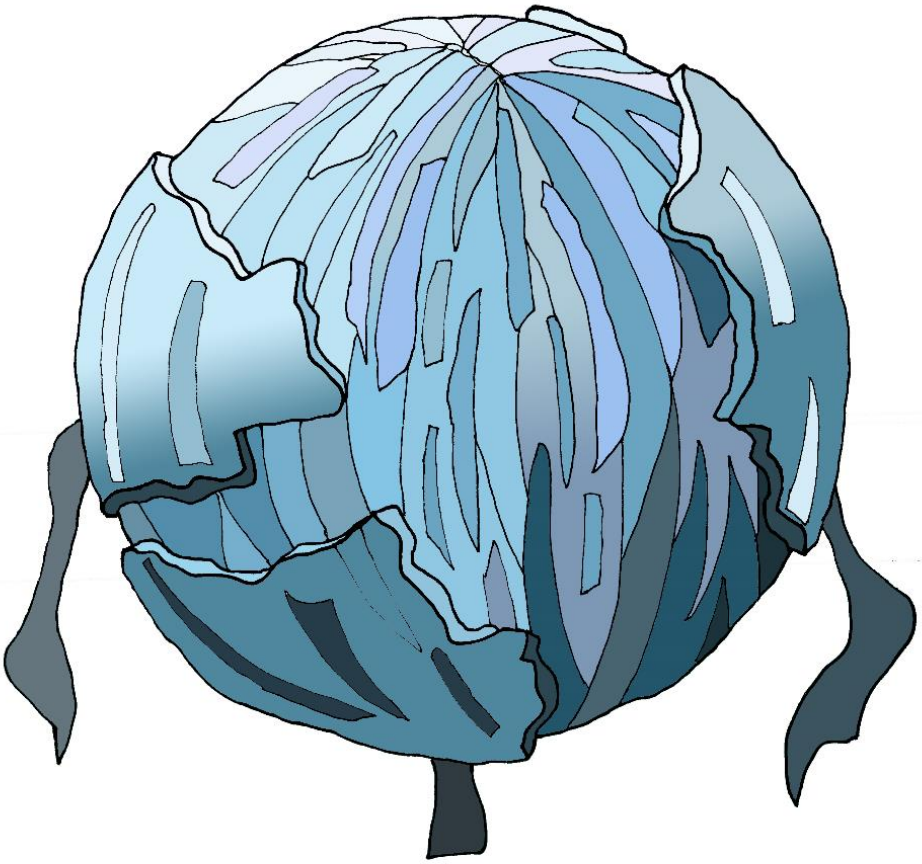

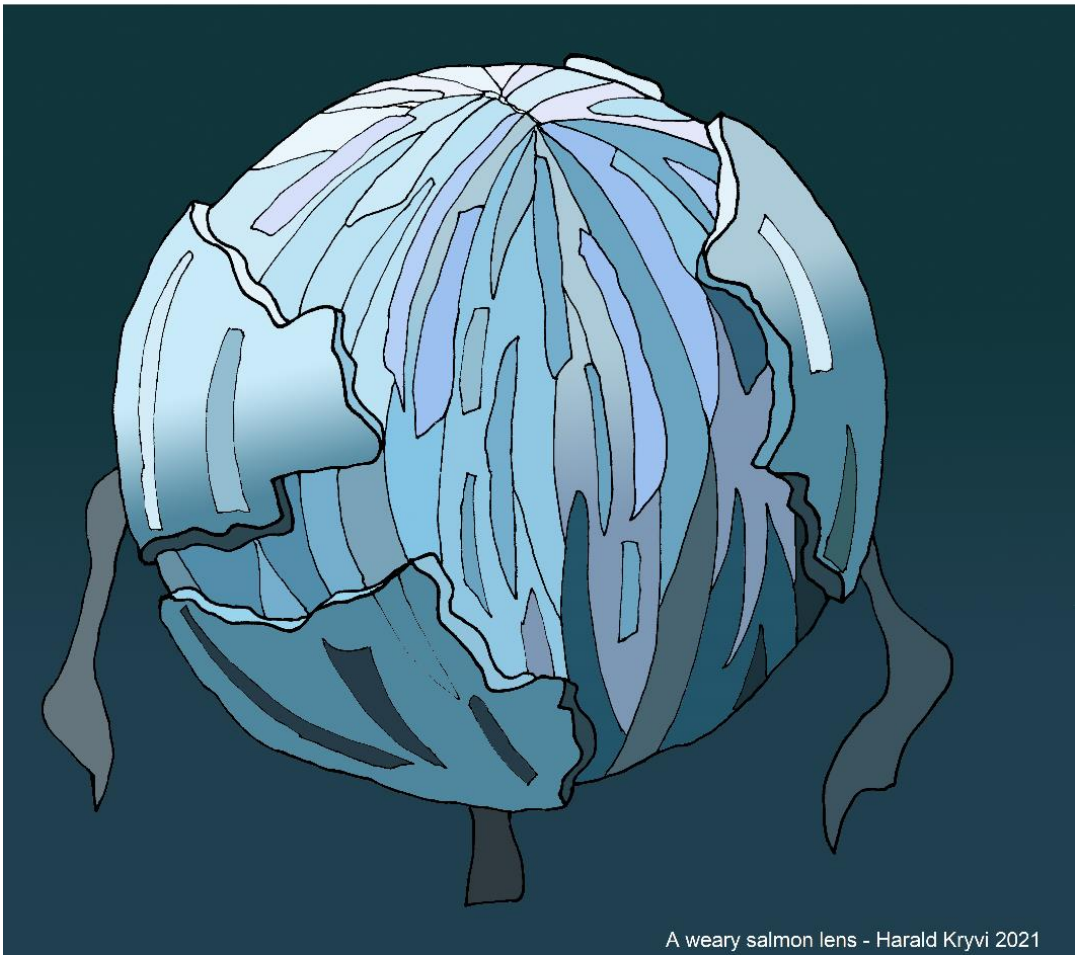
A weary salmon lens - Harald Kryvi 2021

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Onward.

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Abbreviations

Anserine = β-alanyl-N-methylhistidine.

A = Histidine from Ajinomoto Co., Inc.

AM = 1.2% histidine supplemented diets with histidine from Ajinomoto Co., Inc.

AH = 1.4% histidine supplemented diets with histidine from Ajinomoto Co., Inc.

C = Histidine from CJ CheilJedang Corp.

CM = 1.2% histidine supplemented diets with histidine from CJ CheilJedang Corp.

CH = 1.4% histidine supplemented diets with histidine from CJ CheilJedang Corp.

CTRL = The non-supplemented diet given to the control groups.

EEA = Essential amino acids.

His-CD = HIS-containing dipeptides.

L-Histidine = His

K = Histidine from Kyowa Kogyo Ltd.

KH = 1.4% histidine supplemented diets with histidine from Kyowa Kogyo Ltd.

KM = 1.2% histidine supplemented diets with histidine from Kyowa Kogyo Ltd.

KOIs = collective term for Viscera-somatic index – VSI (%), Hepato-somatic index – HIS (%), and Cardio-somatic index CSI (%).

N = sample size.

NAH = N-acetyl-histidine.

Abstract

A comparative nutritional study with post-smolt Atlantic salmon (*Salmo salar* L.) was performed over six weeks to investigate similarities between three suppliers of synthetic His at two dietary levels in a formulated extruded pellet for salmon, and how they affected feed utilization, fish performance, cataract development, digestibility, and biological efficacy. His source or dietary level of His did not affect feed utilization. Digestibility was similar between all experimental groups and the control group. No difference were found in organ indexes for His source, but the dietary level of His showed reduced growth in organ indexes for high supplementation level versus medium and control. Differences existed between experimental groups and the control group for fish performance, but these were assumed to be non-related to nutrition due to their inconsistency. His supplementation reduced cataract severity, while no difference was found between His sources. Tissue levels of His, anserine and NAH concentrations were similar between His sources.

1. Introduction

1.1 Aquaculture

As a critical component of contemporary society, the global food-production industry faces headwinds caused by climate change, population growth, and changing demographics (FAO, 2009, 2017, 2018). In the subsequent years, these headwinds will reshape "*how we eat*" and "*what we eat*" (FAO, 2017) and change the pretension of the consumer with regards to "*needs*" but also "*wants*" (FAO, 2017). As the foremost producer of farmed Atlantic salmon (*Salmo salar L.*) both with regards to volume and revenues, Norwegian aquaculture has come a long way from its novel start. Initially producing 600 Mt in 1974 (Asche & Bjorndal, 2011), primarily by farmers in fjords for niche markets, it has emerged as a massive industry serving a global market (FAO, 2020).

The aquaculture industry participants are not only farmers; they are stewards of our ocean and coast– at any given moment, 400 million salmon and 10s of millions of cleaner fish are in open net pens along the Norwegian coast (Grefsrud, Svåsand, Taranger, & Andersen Berg, 2020), creating a great responsibility for the industry and regulatory authorities. Subpar fish health and welfare is not only unacceptable; it is also expensive (Costello, 2009). The mortality rate of Atlantic salmon transferred to sea in 2016 and has undergone a complete production cycle had a 14% mortality rate (Grefsrud et al., 2019). Increasing mortality strongly correlates with decreased welfare and health for salmon (Grefsrud et al., 2019), and can be caused by an array of different factors (Grefsrud et al., 2019). Still, production-related disorders such as cataract are significant contributors to poor fish health and welfare (Waagbø, 2008), and cataract is found to correlate with dietary deficiency of L-histidine (hereafter called His) (Remø et al., 2014; Waagbø et al., 2010).

1.2 Cataracts in Atlantic salmon

1.2.1 The Historical perspective of cataract in salmon farming

Cataracts are a typical example of a production-related order, i.e., non-infective. Cataract is commonly observed as part of fish pathology, although with varying degrees of severity and prevalence (Bjerkås et al., 2006; Hargis, 1991) and causes observable opacities of the lenses (Bjerkås et al., 2006).

Cataract in salmonids is not a new phenomenon *per se*, reported as early as in the 19$^{th\ century}$ (Roberts, 1989). During the previous three decades (1990 – 2020), two significant shifts caused suboptimal dietary levels of His in salmon feed, increasing the prevalence of cataracts. Two noncorrelated events caused the first. Midway through the 1990s, a consensus among some scientific and commercial groups in Norway suggested that the level of iron in blood meal harmed salmon (Maage, Andersen, & Waagbø, 1994). This [level of iron] caused the removal of blood meal from the salmon feed, even though parts of the scientific community raised their concerns. Later in the 1990s, blood meal was banned from use in salmon feed due to fear of transmitting Bovine Spongiform Encephalopathy (BSE) from ruminants to salmon (European Council, 2002; Wall, 1998), and in turn, the consumer. Bloodmeal is mainly rich in amino acids leucine, lysine, and valine (NRC, 1993). However, compared with fishmeal, it shows higher His and phenylalanine levels with a concentration of 50 grams of His per kilo in spray products (NRC, 1993). These events caused increased cataract prevalence among farmed salmon and increased the disease's focus in scientific communities, which previously had been of little concern to farmers (Wall, 1998). The second significant event was caused by the shift from fishmeal and fish oil to vegetal feed ingredients (Hardy, 2010; Remø et al., 2014). Feed ingredients from vegetal sources do not cover the optimal amino acid for salmon, yet it can be partly achieved by mixing different vegetal feed ingredients (Hardy, 2010). An increase in cataracts in salmon farming in the 1990s increased the scientific focus on the disease (Wall, 1998). Still, it was not until the early 2000s and mid-2010s that scientific work achieved conclusive evidence that the omission of blood meal was the likely cause, and more specifically, insufficient amounts of the essential amino acid His in the salmon feed (Breck et al., 2005; Remø et al., 2014; Waagbø et al., 2010). In 2013, the EU lifted the ban partly by allowing blood meal use from nonruminants in aquafeeds, although the industry is hesitant to use it, perhaps out of fear of negative publicity or consumer perception. By supplementing amino acids to the feed, and although expensive, an optimal level of dietary amino acids in vegetal diets without

blood meal to mitigate nutritional disorders (e.g., cataracts) are achievable (Waagbø et al., 2010).

1.2.2 Cataract as a production-related disorder

The development of opacities is due to changes in epithelial tissue surrounding the lens fibers or the composition and structure of lens fibers (Bjerkås et al., 2006). Initially, the eye's growth was viewed as prioritized over somatic growth in Rainbow trout (Pankhurst & Montgomery, 1994). Later, Breck et al. found that increase of the lens correlated positively with somatic growth in Atlantic salmon, indicating that nutritional deficiencies will not reduce growth before a cataract develops (Breck et al., 2005). Cataract in salmon is, in most cases, first observed as opacities in the anterior or posterior cortical regions or both regions at once. (Breck et al., 2003). Initially, it is observed less frequently in freshwater compared with seawater. In freshwater, cataract is often described as small round-shaped dots in the anterior or equatorial cortex. More severe cataract usually occurs after transfer to seawater (Bjerkås et al., 2006), although it has been observed in freshwater (Bjerkås et al., 2006), especially in triploid salmon (Sambraus et al., 2017). The opacities are often described as having unclear boundaries and a cloudy shape, manifested in the cortical fibers in the anterior polar region and expanding toward the equatorial region (Breck et al., 2003). As the cataract severity increases, the perinuclear region and the cortical region become affected– although nuclear changes are rarely seen (Breck et al., 2003). The lens can limit fiber damage by delimiting whole fibers or ill-affected areas within one fiber (Bjerkås et al., 2006). Such delimiting is frequently found in fish as a lamellar cataract, with non-affected lens fibers surrounding the ill-affected area (Bjerkås et al., 2006). Cataractogenesis in salmon is a severe problem, given that salmon rely almost exclusively on visibility for feed intake. Increasingly, the cataract's severity poses an ethical challenge because the loss of transparency in the lens by cataract could lead to blindness, depending on the severity (Bjerkås et al., 2006).

Furthermore, loss of transparency and resulting blindness caused by cataracts could create economic losses for farmers (Menzies et al., 2002). The lack of ability to use visual sensory organs to feed will reduce feeding rates and potential growth (Ersdal et al., 2001; Menzies et al., 2002; Sveier & Breck, 2001). The losses can increase with increasing development in the production cycle since cataract is not only a problem limited to smolt and post-smolt with

observations of second-year salmon (<1,5 kg) (Waagbø et al., 2010). A reduction in feed and uptake of nutrients could make the salmon more susceptible to secondary infectious disease and increased mortality, thus creating a net loss for the farmers (Menzies et al., 2002).

The cause of cataract is of a multifactorial etiology (Bjerkås et al., 2006; Breck et al., 2005; Remø et al., 2011; Waagbø et al., 2010), and is often related to environmental factors, genetic predisposition, and nutritional factors (Bjerkås et al., 2006; Breck et al., 2005; Remø et al., 2011; Sambraus et al., 2017; Waagbø et al., 2010). The degree of importance for each factor is unknown and is assumed to vary (Sambraus et al., 2017). It is not always easy to pinpoint which specific factor contributes most to cataract outbreaks, as several nutritional and environmental factors can confluence inducing the same final lesion regardless of initial cause (Bjerkås et al., 2006). Thus, being able to separate effects or interactions [or both] of multiple variables is challenging.

Factors that influence cataract development and causes outbreaks in Atlantic salmon are temperature with increased metabolic activity (Bjerkås et al., 2001; Bjerkås & Bjørnestad, 1999; Waagbø et al., 2010), and transfer from freshwater to seawater and during smoltification (Bjerkås et al., 2003; Breck et al., 2005; Breck et al., 2005; Iwata et al., 1987; Jensen et al., 2010; Remø et al., 2014; Sveier & Breck, 2001). Even though specific risk periods exist, cataract development has been observed during freshwater (Bjerkås et al., 1996; Sambraus et al., 2017), as well as in the first (Breck et al., 2003; Remø et al., 2014; Sambraus et al., 2017) and second year in seawater (Waagbø et al., 2010). Failure to develop a functional osmoregulatory system during smoltification in seawater can be a critical factor in inducing cataracts (Bjerkås et al., 2003). Increased osmotic pressure and challenges with smoltification can cause osmotic imbalances in the lens, which causes the formation of cloudiness in the lens that reduces vision (Rhodes et al., 2010; Tröße et al., 2010). In contrast to cataract that appears earlier in freshwater or later during the seawater stage, the osmotically induced cataract is considered reversible (Hargis, 1991). Sambraus et al. 2017 demonstrated the importance of environmental factors regarding mitigating cataract development in freshwater and seawater (Sambraus et al., 2017). Previous studies have also confirmed this by showing that unfavorable temperature conditions could be a factor that affects lens metabolism and cataract development (Waagbø et al., 2010). Adverse conditions for salmon are during periods of fluctuating water temperatures, e.g., the Norwegian summer months, and these periods could lead to several welfare problems such as cataracts (Hevrøy et al., 2013, 2012; Jørgensen et al., 2014; Kullgren et al., 2013). Just how increased or fluctuating temperatures promote cataracts is not known.

Nevertheless, the temperature can cause changes in growth rate and metabolism– thus have an impact on oxidative pressure in addition to nutritional requirements as well as increase the likelihood of cataract developing in Atlantic salmon (Handeland et al., 2008; Jobling, 1994; Remø et al., 2014; Sambraus et al., 2017; Waagbø et al., 2010). As such, functional feeds supplemented with, for example, His may be of even greater importance during periods of elevated water temperatures as utilization increases and the retention rate decreases (Waagbø et al., 2010). Cataract, the eye lens's opaqueness, can also be caused by high stocking density (Calabrese et al., 2017), relevant for land based-farming where a cost-driven focus on increasing stocking density per cage is high (Calabrese et al., 2017). However, studies on halibut indicated that high stocking density did not cause increased cataract development (Remø et al., 2011).

1.3. Functional feeds and production-related disorders

1.3.1 Functional feeds

A vital part of the salmon farming industry is the functional feed components (Waagbø & Remø, 2020). Functional feeds are viewed as part of the solution to the stagnant growth in production volumes and increasing challenges regarding fish health and welfare.

A functional feed is a regular feed supplemented with nutritional additives beyond the requirements, which improves health and welfare (Bellisle et al., 1998; Tacchi et al., 2011; Waagbø & Remø, 2020). Positive implications in salmonids are lower mortality rate, reduced deformities and disorder frequencies, and more efficient production cycles (Waagbø, 2006). Functional feeds include, but are not limited to, probiotic bacteria, prebiotic components, immunostimulants, enzymes, antioxidants, vitamins, organic acids, and plant extracts (Gatlin, 2007). Modern feed using other protein sources than fishmeal are likely to benefit from supplementing functional components. The increase in production-related disorders in salmon farming and the possibility of function feeds to reduce the prevalence of production-related disorders highlights its importance (Waagbø, 2008). For example, vegetal ingredients tend to have insufficient essential amino acids such as His to cover the dietary levels needed to mitigate the production-related disorder cataract, and His as a functional additive mitigates the problem, although at an economical cost (Breck et al., 2005; Remø et al., 2014; Sambraus et al., 2017; Tröβe et al., 2010; Waagbø et al., 2010). Nonetheless, supplementation of functional feed ingredients, in general, can positively impact the cost equation (Waagbø & Remø, 2020).

1.4 Ocular anatomy and physiology

1.4.1 Anatomy

The fish lens is an avascular organ, and to a great extent, all light refraction in the teleost eye occurs in the lens, which enables the concentration of the light on the retina (Kryvi & Poppe, 2016). Besides a minority of teleosts living inside dark caverns, most teleost species rely heavily on vision to locate food; thus, enabling greater visuality through light refraction on the lens is (Kryvi & Poppe, 2016). Therefore, transparency is needed, and it manages transparency by maintaining a default structure of lens proteins, default arrangement of lens cells and fibers, and keeping fibers in a moderately dehydrated state (Bjerkås et al., 2006). Like other vertebrates, the Atlantic salmon lens receives nourishment from the ciliary epithelium's aqueous humor (Midtlyng et al., 1999). The deeper lens fibers have, for the most part, lost their ability to synthesize proteins, and therefore crystalline is synthesized in the epithelial and superficial cortical cells (Bjerkås et al., 2006). The inactivity of the deeper lens fibers is why the irreversible part of cataract development is seen in salmon (Bjerkås et al., 2006).

1.4.2 Physiology

Atlantic salmon faces increasing osmotic pressure in the lens during seawater transfer (Bjerkås et al., 2006). The ocular humor tries to achieve and maintain homeostasis by using various independent regulating mechanisms that also have essential roles in preserving integrity (Handeland et al., 1998). These regulating mechanisms include pH buffering and a continuous adjusting of the volume of humor in the lens (Mathias et al., 1997). Besides having essential multiple enzymatic and non-enzymatic systems whose purpose is to reduce or eliminate risk and damage caused by external factors such as, e.g., oxidative stress, the lens fibers themselves are in most cases held in a state of dehydration. Mitigating external risk factors is possible due to actions driven by Na^+/K^+-pumps (Mathias et al., 1997). The development of cataracts can impair the function of Na^+/K^+-ATPase (Spector', 1995). Such impairment can propel the lens to swell due to either failed mitigation of building osmotic pressure or preclusion of volume mechanism (Jacob, 1999). If the ocular lens failed to regulate crystallin's optimal volume, it could severely affect transparency (Donaldson et al., 2009). If under continuous pressure, permanent damage and problems such as protein aggregation, precipitation, and opacification follow (Jacob, 1999).

The lens's cellular membranes are transmembrane solvent channels known as aquaporins (AQP), which promote intracellular fluid homeostasis (Németh-Cahalan & Hall, 2000). Both AQP0 and AQP1 have previous studies showing them to act as peroxiporins, facilitating membrane transport of hydrogen peroxide (Varadaraj & Kumari, 2020). If these proteins are missing or defect from an otherwise standard lens under any circumstances, a cataract will develop (Németh-Cahalan et al., 2004). AQPO achieves intracellular fluid homeostasis, regulated by Ca^{2+} and pH (Németh-Cahalan et al., 2004; Varadaraj et al., 2005). In studies on mammalians, AQP0 channels have His bound to them at specific positions, enabling them to act as pH sensors (Varadaraj et al., 2005). Studies on reptiles have identified the same pairing of AQP0 and His, but the His has been at different positions than mammals (Ringvold et al., 2003). Thus, a possibility that the position of and amino acid sequence could affect the lens pH sensitivity is viable. However, other unknown components involved in lens biochemistry could also be present (Bjerkås et al., 2006).

Most of the energy obtained by the lens arrives in the form of glucose. Fatty acids and amino acids can be sources, although only minor than glucose (Brown & Bron, 1995). Specific transporter molecules present both on the lens surface and in the deeper parts of fiber membranes transport glucose into the lens (Lucas & Zigler, 1988). Once the glucose is inside the lens, the utilization will occur either by glycolysis, regulated by intracellular calcium levels, the pentose pathway (hexose monophosphate shunt), or the sorbitol pathway. An increase in glucose would cause the accumulation of sorbitol in the lens, causing an increase in water uptake by osmosis, which would lead to swelling of the lens fibers and the development of cataracts (Basher & Roberts, 1995). Unfortunately, sorbitol's biochemical characteristics and functions in salmonids are unknown (Bjerkås et al., 2006).

1.5 Histidine and its imidazoles

1.5.1 Histidine

Previously, sup-optimal levels of methionine, tryptophan, riboflavin, zinc, manganese, and cataracts were linked (Bjerkås et al., 2006). Nonetheless, in recent years a coherency between cataract and low dietary levels of the essential amino acid His in salmon has been reported

(Bjerkås et al., 2006; Breck et al., 2005; Remø et al., 2014; Waagbø et al., 2010). His is an essential amino acid for protein synthesis. It also inhabits important biochemical and metabolic roles in free form in high concentrations in salmonid tissues (Waagbø et al., 2010). The free imidazole form of His, known as the metabolite NAH that the lens synthesizes, has been identified as the key His compound for mitigating cataracts (Breck et al., 2005a; Breck et al., 2005b). The metabolite NAH is not only present in lenses found both in the heart and brain of salmonids (Breck et al., 2005; Breck et al., 2005). Dipeptide anserine, another major His imidazole in salmonids, is most abundant in salmonids' white muscle (Remø et al., 2014). Previous studies have shown that His supplementation elevates concentrations of NAH and dipeptide anserine in salmonids and reductions in cataract outbreaks and severity in salmonids (Breck et al., 2005; Remø et al., 2014; Waagbø et al., 2010). His has been shown to influence anti-inflammatory functions in human and animal model studies (Yoneda et al., 2009). His effects on apoptosis regulation, cell growth, and antibody production are hypothesized to be linked to its antioxidative characteristics (Li et al., 2007). Studies on salmon found that supplementation of His ex vivo indicated it could affect apoptosis and the antioxidant system (Remø et al., 2011).

The original requirements of His to sustain protein synthesis and growth (0.8% His/kg feed) (NRC, 2011; Scott, 1998) does not meet the required amount of His for tissue buffering, antioxidative measures, and osmoregulatory components (Remø et al., 2014). Remo et al. (2014) estimated the optimal dietary level of His at 1.34% His/kg feed, well above the requirements to sustain protein synthesis and growth. Exaggerated His additives in feed can do more harm than good. Excess amounts of His added to feed could cause the initiation of the enzyme “histidase.” The enzyme enables His degradation, causing an imbalanced amino acid composition in the diet and increased catabolism (Holecek, 2020). Furthermore, when composing feed diets with His, it is vital to keep in mind that His can bind and modulate the absorption of Zn, Cu, and Fe (Wade & Tucker, 1998). Thus it could potentially affect the distribution and excretion of elements in the Atlantic salmon, meaning His may affect the tissue distribution of elements (Remø et al., 2014).

1.5.2 N-acetyl-histidine - NAH

The underlying mechanism for cataract mitigative characteristic of His is assumed to be the His metabolite N-acetyl-histidine (hereafter called NAH) found in the lens, the heart, and the brain of Atlantic salmon (Bjerkås et al., 2006; Breck et al., 2005; Remø et al., 2014; Remø et al., 2011; Rhodes et al., 2010; Waagbø et al., 2010). Its functions include contributing to water balance as an osmolyte (Baslow, 1998; Rhodes et al., 2010), buffer component (Breck et al., 2005), detoxification of cytotoxic-reactive carbonyl species (Aldini et al., 2002; Hipkiss & Brownson, 2000), and as an intracellular antioxidant (Remø et al., 2011). As a result, the metabolite NAH is essential for preserving lens water balance and cell integrity. Multiple studies suggest a correlation between cataracts and oxidative stress, including human and animal studies (Vinson, 2006; Williams, 2006). Furthermore, studies on Atlantic Salmon indicate that a balanced pro- and antioxidant level in diets can reduce cataracts' prevalence (Waagbø et al., 2003). Indeed, His imidazoles in humans have shown abilities to perform antioxidative functions, e.g., carnosine (Babizhayev et al., 2004). Thus promoting the case for NAH as analog in salmonids performing the same antioxidative activities and mitigating oxidative stress.

After transfer to seawater, NAH initiates a vital role as an osmolyte mitigating the increased osmotic pressure on the Atlantic salmon lens (Breck et al., 2005; Sambraus et al., 2017). For any osmolyte, it must be able to react diversely to any form of osmotic stress (Rhodes et al., 2010). NAH inhabits such characteristics and is viewed as the primary osmolyte, which balances extracellular osmolality in Atlantic salmon lenses (Rhodes et al., 2010). As for most animal cells, water influx and efflux in teleost lenses are achieved by aquaporin channels (Rhodes et al., 2010). In general, when cells are faced with osmotic changes, they allow the transfer of ions, osmolytes, and water over the plasma membrane (Hoffmann et al., 2009). The cell volume of teleost lenses is determined by two factors: the extracellular osmolality, and second, the cells' content of osmolytes inhabits osmotic activity (Rhodes et al., 2010). By utilizing osmolytes that do not interfere with normal cell functions, the lenses avoid maintaining large ion concentrations in their intracellular milieu during hyperosmotic conditions (Hoffmann et al., 2009). Nevertheless, NAH does not seem to be a molecular water pump due to the lens's high water permeability (Baslow, 1998; Rhodes et al., 2010). Rhodes et al. (2010) hypothesized that NAH was a key osmolyte due to the correlations between in vitro and in vivo concentrations of NAH and the external medium's osmolality. In similar studies, Tröße et al. (2009) identified the efflux of NAH from Atlantic salmon lenses ex vivo during hypo-osmotic conditions correlated with dietary His content and protein sources. Earlier studies have indicated that the

amount of NAH synthesized in freshwater was lower than seawater and that cataract development was negligible (Breck et al., 2005; Remø et al., 2014; Taylor et al., 2015). In more recent studies, however, it has been shown that NAH synthesis initiates in freshwater and that the development of severe cataracts is plausible given higher temperatures (Sambraus et al., 2017), emphasizing the importance of environmental factors with regards to cataract. In previous studies, NAH in the lens has been shown to correlate positively with His amount added to feed and negatively with cataract development (Breck et al., 2005; Remø et al., 2014; Waagbø et al., 2010). Previous studies have also shown that salmonids priorities biosynthesis of anserine over NAH, even during periods of critical His supply and cataract status (Waagbø et al., 2010). NAH as an indicator of cataract susceptibility is also deemed plausible (Remø et al., 2014; Waagbø et al., 2010).

1.5.3 β-alanyl-N-methylhistidine - Anserine

Myotomes, which are separated by myosepta (Merriam-Webster, 2020), have regular arrays of actin (I-filaments) and myosin (A-filaments) on sarcomeres in adjacent myofibrils (Johnston et al., 2006). The myotomes of teleost fish contain distinct layers of slow (red) and fast muscle (white) that power slow and high-speed swimming, respectively (Johnston et al., 1977). Like pelagic fish species such as scombroids, salmon rely on His and its derived dipeptides to buffer protons produced by high anaerobic activity in white muscle tissue (Suzuki et al., 1990; Suzuki et al., 1987). Anserine is an essential buffer that maintains intracellular pH homeostasis, needed after laborious exercise during river migration to seawater and burst swimming (Abe, 1987, 1995; Ogata & Murai, 1994; Ogata, 2002; Ogata et al., 1998; Okuma & Abe, 1992). Of all the known amino acid chains in proteins, only the imidazole ring of His has been identified as applicable to function as a proper pH buffer to maintain intracellular pH homeostasis (Derave et al., 2010). pH-buffering abilities are regardless of which nitrogen in the imidazole ring, which it uses, releasing protons to create acid or base form (Holecek, 2020). Anserine differs from His in that it inhabits a constant buffer capacity along with the rearing temperature range in salmonid farming (Abe & Okuma, 1991). The estimation of the pKa-value of an imidazole ring of His is 6.2, bound to proteins 6.5 and as anserine 7.1 (Abe, 2000). Hence, His-CDs such as anserine can act as forceful buffers and mitigate changes in intracellular pH in salmonid tissues during anaerobic exercises such as river migration or burst swimming. Sambraus et al. (2017) identified the level of anserine at the end of the freshwater period as elevated in groups feed

His-rich diets, and thus showing the same positive correlation as NAH with regards to the amount of His in feed and concentrations of metabolites in the salmon. Observations of the correlation in previous studies are recognized, where anserine concentration in both freshwater and seawater positively correlated with the amount of dietary His (Remø et al., 2014). In previous studies, anserine concentrations in freshwater were low. After transfer to seawater, the synthesis of anserine seemingly increased (Breck et al., 2005; Ogata et al., 1998). Sambraus et al. (2017) hypothesized that due to evidence of anserine synthesizing in high concentrations in freshwater before smoltification, a case for a correlation between anserine synthesis and the size of the fish could be made. Meaning a threshold could exist whereby fish can start synthesizing anserine and NAH synthesis before the fish are transferred to sea and before smoltification (Sambraus et al., 2017). However, more recent studies found no correlation between salmon size and its ability to synthesize His imidazole's in fresh water (Slaatmo, 2021).

1.6 Aquaculture feed legislation

1.6.1 The legal framework for production and use of L-histidine

After the use of bloodmeal was removed from aquafeed due to fear of toxic iron levels for salmon (Maage et al., 1994), and later banned due to fear of Bovine spongiform encephalopathy (Wall, 1998). In addition to the reduction of His-rich fishmeal in the salmon feed (Aas, Ytrestøyl, & Åsgård, 2019; Hardy, 2010; Ytrestøyl, Aas, & Åsgård, 2015), new sources of His to support tissue His status and cataract mitigation in Atlantic salmon was needed. As His is one of the ten essential amino acids, an external source is needed as an additive in aquafeed. L-Histidine monohydrochloride monohydrate, a synthetic form of L-His, can be obtained by fermentation of an isolated strain of bacteria such as *Escherichia Coli* or *Corynebacterium glutamicum* (EFSA FEEDAP Panel, 2004, 2019a, 2019b). After fermentation, further isolation from the broth, decolorization, concentration, crystallization, and drying is needed (EFSA FEEDAP Panel, 2004).

Assessment of feed additive's effect on salmon is challenging due to their multidimensional nature, and therefore, it is vital to have a comprehensive legal framework ensuring quality requirements are met (Waagbø & Remø, 2020). The European Food Safety Authority (hereafter

referred to as “EFSA”) is the central European agency for the admittance of new additives for aquafeed in Europe (European Parliament and Council, 2002). Before adding new additives to aquafeed, it must be authorized by complying with the directive's provision (Gasco et al., 2018). To obtain authorization, a producer must submit a dossier with complete compositional profiles containing relevant data and studies that can demonstrate the product's efficacy and safety for animals, consumers, and the environment (Gasco et al., 2018). The authorization of new synthetic forms of His was previously reviewed by the legislation of “Council Directive 82/471/ECC concerning certain products used in animal nutrition” (European Parliament and Council, 1982). The directive concerns products such as His produced with specific technical processes and are additives to feeds. Member States are only allowed to add or market His products prescribed to one of the groups listed in the annex or appear in the annex, and the conditions laid down therein are fulfilled (European Parliament and Council, 1982). After the implementation of Regulation “(EC) No 1831/2003 on additives for use in animal nutrition”, amino acids previously covered by Council Directive 82/471/ECC of June 1982 concerning products used in animal nutrition, was thereinafter included as a category of feed additives—thus transferred from the scope of “Council Directive 82/471/ECC” to “(EC) No 1831/2003” (European Parliament and Council, 2003). Regulation (EC) No 1831/2003 constitutes the legal framework that governs the community authorization of additives for use in animal nutrition (European Parliament and Council, 2003). In particular, Article 4(1) of that regulation states that “any person seeking authorization for feed additive or new use of a feed additive shall apply per article 7” (European Parliament and Council, 2003). Article 7(1) of Regulation (EC) No 1831/2003 states that the application shall be forwarded to the European Food Safety Authority (EFSA) as an application under Article 4(1). According to article 8 of Regulation (EC) 1831/2003, EFSA, after confirming the particulars of the applicant's documents, shall undertake assessments to determine whether the feed additive complies with conditions laid down in article 5. The EFSA shall conclude whether the safety of target species, consumer, user, and the environment is satisfactory and whether the product's efficacy is satisfactory. Also, article 14(1) of that regulation states that “an application of renewal shall be sent to the commission at the latest one year before the expiry date of authorization” (European Parliament and Council, 2003).

1.6.2 Suppliers of L-histidine for aquafeed

The Japan, Tokyo-based company - Kyowa Kogyo Ltd. - sent an application for authorization of L-Histidine monohydrochloride monohydrate to the EFSA in 2004. The His was a synthetic form, obtained by fermentation of an isolated strain of *Escherichia Coli* ATCC 21318, for use in aquafeed for salmon (EFSA FEEDAP Panel, 2004). The EFSA received the application in 2004, and as this was before the implementation of (EC) No 1831/2003, the Council Directive 82/471/ECC was used to review the application from Kyowa Kogyo Ltd. (EFSA FEEDAP Panel, 2004). The product's chemical formula is $C_3H_3N_2$-CH_2- CH(NH_2)-COOH·HCl·H_20. The molecular weight 209.63 Da, with L-histidine of 74%, equivalent to approximately 100% L-Histidine·HCl·H_2O_2. EFSA created a FEEPAP panel to assess the product, safety of target animals, the worker, the user, the consumer, the environment, and the bioavailability with support from previous known information and information submitted by the applicant. The FEEDAP Panel concluded that L-Histidine monohydrochloride monohydrate from Kyowa Kogyo Ltd. had "No adverse effect on fish growth or health" and "… does not expect a risk to the consumer" (EFSA FEEDAP Panel, 2004). Furthermore, "the bioavailability … is as high as that of histidine from natural sources" and "… no adversely influence on the environment" (EFSA FEEDAP Panel, 2004). Thus, EFSA granted authorization for the use of L-histidine monohydrochloride monohydrate from Kyowa Kogyo Ltd. with the concluding remarks ".... a source of synthetic histidine able to replace histidine from natural sources"(EFSA FEEDAP Panel, 2004).

In 2019, Ajinomoto Animal Nutrition Europe, a subsidiary of the Japan, Tokyo-based company - Ajinomoto Co., Inc. - sent an application for authorization of L-histidine monohydrochloride monohydrate, a synthetic form of His, obtained by fermentation of an isolated strain of *Escherichia Coli* NITE BP 022526, for use as "a feed additive for all animals" (EFSA FEEDAP Panel, 2019a). The EFSA received the application in 2019, and as this was after the implementation of (EC) No 1831/2003, the EFSA reviewed the application using (EC) No 1831/2003 (EFSA FEEDAP Panel, 2019a). The product's chemical formula is $C_6H_9N_3O_2$·HCl·H_2O(EFSA FEEDAP Panel, 2019a). The molecular weight 209.63 Da, with L-histidine of 74.1% (range 72.3 – 74.7%), equivalent to approximately 100% L-Histidine·HCl·H_2O_2 (EFSA FEEDAP Panel, 2019a). To comply with article 8 of Regulation (EC) No 1831/2003, EFSA constructed a FEEPAP panel to assess whether the product complies with the conditions stated in article number 5; safety of target animals, the worker, the user, the

consumer, and the environment, and the bioavailability with help from previous known information and information submitted by the applicant. The FEEDAP panel notes that "… using E. coli (NITE BP 02526) does not give rise to any safety concern regarding the production strain and its genetic modification". Furthermore, it concludes that "The use of L-histidine … is safe for target species when used as a nutritional additive to supplement diet in appropriate amounts …", and "… supplemented at levels appropriate for the requirements of target species is considered safe for the consumer.". It is not "irritant to skin or eyes, nor a skin sensitizer", However, "there is a risk for persons handing the additive from exposure to endotoxins by inhalation.". L-Histidine monohydrochloride monohydrate from Ajinomoto Animal Nutrition Europe does not "represent a risk to the environment" and is viewed as "an efficacious source of the essential amino acid L-histidine for non-ruminant animal species". Thus, EFSA granted authorisation for the use of L-histidine monohydrochloride monohydrate from Ajinomoto Animal Nutrition Europe.

In the same period, CJ Europe GmbH, a subsidiary of the South Korea, Seoul-based - CJ CheilJedang Corp. - sent an application for authorization of L-histidine monohydrochloride monohydrate, a synthetic form of His, obtained by fermentation of an isolated strain of *Corynebacterium glutamicum* KCCM 80179 for use as "… intended to be used in feed and water for drinking for all animal species as a nutritional additive …" (EFSA FEEDAP Panel, 2019b). The product's chemical formula is $C_3H_3N_2–CH_2–CH(NH_2)–COOH\cdot HCl\cdot H2O$ (EFSA FEEDAP Panel, 2019b). The molecular weight 209.63 g/mol, with L-histidine of 73.5% 'as is' (range 73.5–73.6%), ≤ 10% moisture, and ≤ 1% ash, equivalent to ≥ 98% L-histidine monohydrochloride monohydrate L-Histidine·HCl·H_2O_2 (EFSA FEEDAP Panel, 2019b). To comply with article 8 of Regulation (EC) No 1831/2003, EFSA constructed a FEEPAP panel to assess whether the product complies with the conditions stated in article number 5; safety of target animals, the worker, the user, the consumer, and the environment and the bioavailability with help from previous known information and information submitted by the applicant. The FEEDAP panel remarks that "The product under assessment is produced by fermentation using a strain C. glutamicum (KCCM 80179) which fulfills the QPS qualifications for production purposes" (EFSA FEEDAP Panel, 2019b). Furthermore, the panel concludes that L-Histidine monohydrochloride monohydrate from CJ Europe GmbH is "… safe for the target species when used as a nutritional additive to supplement the diet in appropriate amounts …" and "No physicochemical incompatibilities in the feed are expected with other additives, medicinal products or other feed materials." (EFSA FEEDAP Panel, 2019b). Also, it "… is considered

safe for the consumer" and "… using C. glutamicum KCCM 80179 is considered not irritant to skin, is a mildly irritant to eyes, and it is not a skin sensitiser." (EFSA FEEDAP Panel, 2019b). Furthermore, it "… C. glutamicum KCCM 80179 in animal nutrition is not expected to represent a risk to the environment." and " … is considered as an efficacious source of the essential amino acid L-histidine for non-ruminant animal species" (EFSA FEEDAP Panel, 2019b). Thus, EFSA granted authorization to use L-histidine monohydrochloride monohydrate from CJ Europe GmbH as a nutritional additive for all animal species (EFSA FEEDAP Panel, 2019b).

1.7 The aim of the study

The present study aimed to investigate the efficacy of new synthetic sources of L-Histidine monohydrochloride monohydrate to support tissue His status and cataract mitigation in Atlantic salmon smolt after sea transfer by using up-to-date industrial diets.

This included:

1. Evaluate whether the FCR, feed intake, somatic weight, SGR, K-factor, and somatic indexes differed between fish given the three His sources.

2. Compare the His source's ability to reduce cataract prevalence and severity and evaluate the necessity of high His supplementation levels for optimal cataract mitigation.

3. Investigate whether the lens and heart tissue levels of His and NAH, and muscle tissue levels of His, anserine, carnosine, β-alanine, and 1-methyl-histidine correlated with His supplementation levels and whether the tissue levels differed between the His sources.

2. Material and methods

2.1 Experimental design and diets

2.1.1 Design and production of experimental diets

Seven diets were formulated by Mowi Feed (Bergen, Norway), one control diet without His supplementation, and six diets containing L-histidine monohydrate from three different suppliers, *ceteris paribus*. All diets reflected up-to-date industry-standard recipes except for His supplementation. Formulation and chemical composition for each diet is given in *Tables 1* and *2*. Vitamins and minerals were added to all diets to reflect industry standards and fulfill the National Research Council recommendations (NRC, 2011). Protein level reflected industry standards and was above the requirements in all diets (NRC, 2011). The production of feeds was executed by Nofima (Bergen, Norway). The diets were extruded to give pellets 3,3 mm in diameter.

The supplemented His was added in crystalline form, and the feeds collectively called “the experimental feeds and groups.” The non-supplemented diet (assumed 0.8% His of diet d.w.from the feed ingredients) was used as control, as this was viewed as a basic level of His below the amount needed to mitigate cataract development in post-smolt Atlantic salmon. The non-supplemented diet was called “CTRL,” while experimental diets with medium (1.2% His of diet d.w. target level) or high (1.4% His of diet d.w. target level) content was named after their His source producer and level; Kyowa Kogyo Ltd., (named "KM" or "KH"), Ajinomoto Animal Nutrition Europe (named "AM" or "AH") or CJ CheilJedang Corp (named "CM" or "CH") (experimental groups; AM, AH, CM, CH, KM and AH). Yttrium oxide (Y_2O_3) was added to the feed as an inert marker to assess dietary His digestibility.

Table 1 Feed formulation in the control and the 6 experimental diets. Control: 0.8 % His of diet d.w., KM: 1.2% His of diet d.w. , KH: 1.4% His of diet d.w. , CM: 1.2% His of diet d.w. , CH: 1.4% His of diet d.w., AM: 1.2% His of diet d.w. , AH: 1.4% His of diet d.w.

Ingredients and nutrients composition g kg $^{-1}$	CTRL	KM	KH	CM	CH	AM	AH
Feed formulation							
Fish meal NE Atlantic	20,7	20,7	20,7	20,7	20,7	20,7	20,7
Fish oil NE Atlantic	17,0	17,0	17,0	17,0	17,0	17,0	17,0
Soy protein concentrate	11,0	11,0	11,0	11,0	11,0	11,0	11,0
Corn gluten meal	10,0	10,0	10,0	10,0	10,0	10,0	10,0
Vital wheat gluten	15,2	15,2	15,2	15,2	15,2	15,2	15,2
Rapeseed oil, crude degummed	4,9	4,9	4,9	4,9	4,9	4,9	4,9
Rapeseed oil, high lecithin	1,0	1,0	1,0	1,0	1,0	1,0	1,0
Wheat whole	11,5	11,1	10,9	11,1	10,9	11,1	10,9
Vitamins, minerals and amino acids[1]	4,7	4,7	4,7	4,7	4,7	4,7	4,7
Kiowa L-HIS		0,36	0,59				
CJ L- HIS				0,36	0,58		
Ajimoto L-HIS						0,37	0,60
Water	4,1	4,1	4,1	4,1	4,1	4,1	4,1

[1] Vitamin, minerals and amino acids; Mowi Feed, Bergen, Norway (fulfilling recommendations for salmonids given by (NRC, 2011).

Table 2 Proximate composition (g kg^{-1}) and indispensable amino acid (g/16N) in the control and the 6 experimental diets. Control: 0.8 % His of diet d.w., KM: 1.2% His of diet d.w. , KH: 1.4% His of diet d.w. , CM: 1.2% His of diet d.w. , CH: 1.4% His of diet d.w., AM: 1.2% His of diet d.w. , AH: 1.4% His of diet d.w.

Ingredients and nutrients composition g kg $^{-1}$		Control	KM	KH	CM	CH	AM	AH
Proximate composition (digestible nutrients[1])								
Crude protein, %		43,7	44	43,5	43,8	44,3	44,5	44,1
Dry matter, %		89,8	89,3	89,7	89,1	89,6	89,9	88,8
Crude fat, %		26,3	24,8	26,6	25,5	25,8	25,7	25,6
Ash, %		5,6	5,7	5,6	5,5	5,5	5,5	5,4
Starch & sugar, %		10,5	10,1	10,6	10,3	9,8	10,3	9,3
Crude fibre, %		1,2	1,2	1,4	1,3	1,2	1,3	1
Amino acids (g/16gN)	AA requirement[2]							
Histidine, %	1,0 - 1,8	0,93	1,20	1,50	1,20	1,30	1,20	1,30
Histidine , %, dw		1,04	1,34	1,67	1,35	1,45	1,33	1,46

[1] The analysed composition of diets multiplied by the measured digestibility for the respective nutrients, given in parenthesis behind the values for analysed composition.

[2] Data from (NRC 2011), requirement are given as % of the AA of CP, which corresponds to g/16gN.

2.1.2 Experimental design

The 6-week nutritional trial was carried out at IMR, Matre Research Station, from 16 December 2020 to 27 January 2021, according to Norwegian (FOR-2015-06−18-761) and European (Directive 2010/63/EU) research legislation.

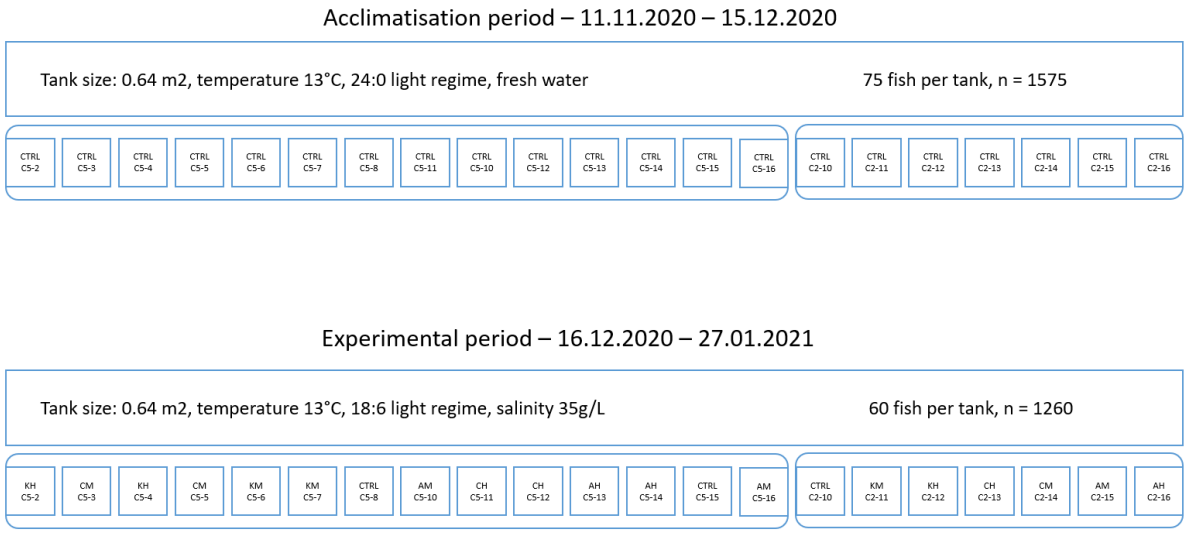

Fig 1. Experimental design of the nutritional trial. 1575 fish divided into 21 tanks underwent 4 weeks of acclimatization before a reduction of 15 fish per tank and transferred to seawater, and after that fed one of 6 experimental diets or a control diet in triplicates for six weeks.

The fish were produced by standard production methods at Matre Research Station and fed commercial diets *ad libitum* before the acclimatization period. The parr-smolt transformation was initiated using a continuous light regime (OSRAML 18 W/840 LUMILUX, OSRAM GmbH, Ausburg, Germany) before the experiment and confirmed using a standard seawater test before the experimental start. The fish were acclimatized in the tanks and given the CTRL diet for four weeks before the nutritional study commenced. Unvaccinated salmon parr (*Salmo salar L.*, Aquagen strain) with an average weight of 126g were randomly distributed among 21 fiberglass tanks with approximately 0.64m^3 freshwater volume after sorting out very large or small salmon parr, or salmon parr with deformities or injuries. Water flow in the tanks was approximately 10 – 12 L/min, and the water temperature was kept constant and averaged 13°C.

At the start of the experiment, the amount of fish in each tank was reduced from 75 to 60 to accommodate for biomass increase, sampling, and seawater challenge, and randomly assigned one of the seven diets in triplicates. The average weight as biomass divided by the number of

fish was 178g (SD 12g, n = 21). The smolt was put on seawater with a salinity of 35 g/l, 100% dissolved oxygen (DO), a mean temperature of 13°C, and exposed to an 18:6 light regime (daylight tubes OSRAML 18 W/840 LUMILUX, OSRAM GmbH, Ausburg, Germany, producing 960 lux were positioned under the roof of the tank). Triplicate groups of salmon smolt were fed six experimental diets delivered as extruded pellets. The fish were fed between the hours of 09:00-10:30 and 12:00-13:30 using automatic feeders (ARVO-TEC T Drum 2000, Arvotec). The feeding regimes were slightly overfeeding (1.5% of the biomass), and excess feed was collected within 15 minutes after the termination of each meal to calculate daily feed intake, as described by (Helland et al., 1996). An internally operated computer system managed both light and feeding regimes (Normatic AS, Nordfjordeid, Norway). Mortality was controlled daily, with dead fish removed and counted before being ensiled. After six weeks, the experimental period was terminated with the final sampling.

2.2 Sampling procedures

The initial sampling was conducted before the sea water phase. A second and final sampling was conducted at the end of the experimental period. Samplings were standardized by starving the salmon smolts for approximately 24 hours preceding each sampling.

The total biomass in each tank was determined before the trial and at the end of the trial. Feed samples and feces were collected for all experimental diet groups and analyzed for feed composition and digestibility using accredited methods.

All samplings were initiated with smolts being euthanized with overdose anesthesia (Finquel), chosen randomly from the experimental tanks. The weight (to the nearest 1 gram) and length (to the nearest 1 millimeter) were quantified and recorded. Qualitative measurement of cataract severity was assessed using cataract scoring methods described by (Wall & Bjerkas, 1999) and using a biomicroscope (HEINE Optotechnik GmbH & Co. KG, Herrsching, Germany). Each salmon lens was given an individual score of 0 – 4, reflecting the degree of opacification, and the two scorings per salmon were summarized for a total score of 0 – 8 per fish. At the initial sampling, 64 fish were scored, and at the termination, 30 fish per tank was scored.

Samples of lens tissue were collected by carefully dissecting the lens after opening the cornea by an incision along the limbus and removing surface liquid on the lens. Lens tissue was

sampled for determination of NAH and His. Hearts were sampled for analysis of NAH and His concentrations. Skin-free epaxial white muscle tissue below the dorsal fin was sampled to quantify total free amino acid concentration. At the initial sampling, 20 fish were sampled for tissues. At the termination, blood and tissue samples were collected from six fish from each tank.

At the feed trial termination, all fish within each tank was stripped for feces by gently squeezing the latter half of the hindgut to avoid contamination by urine (Austreng, 1978). The hindgut content was collected and frozen at -20°C until the dry matter nitrogen content analysis was determined at Nofima (Bergen, Norway). All samples collected were flash frozen and transported on dry ice and stored at – 80°C for further analysis.

2.3 Feces and feed analysis and composition

Feces and diets were analyzed by Nofima (Bergen, Norway) using accredited European Union Standard Method for crude protein (Kjeldahl-Nx6.25; EU Dir 93/28/EEC), crude fat (Method B in Dir 98/64/EC), dry matter (EU Dir 71/393/EEC) and ash (EU Dir 71/250/ECC).

2.4 Blood sampling and analysis

By methods described by (Sandneset al., 1988), blood was sampled from the caudal vein. Approximately 1,5 mL of blood was sampled from each fish during initial sampling (n = 20) and at the final sampling (n = x) and stored in a plastic tube kept at 4°C. The plastic tubes were then centrifugated at 3000 g for two minutes, and the plasma was collected and transferred to 1,5 mL plastic tubes and stored on ice. An ABL 9180 Analyzer (Dialogue Diagnostics LLC, Ukraine) assessed plasma chloride concentration using an ion-selective electrode.

2.5 Determination of histidine and histidine imidazole concentrations

2.5.1 Heart and lens NAH concentrations

By utilizing Waters Reverse-phase High-performance Liquid Chromatography (HPLC) (Waters Corp. Milford, MA, USA) based on methods described by (O'Dowd, Cairns, Trainor, Robins, & Miller, 1990) and slightly modified by (Breck et al., 2005) –heart and lens NAH concentrations were quantified. Lenses and heart tissue from each of the 18 experimental groups and the 3 control groups were analyzed (3 pooled samples per diet), in addition to lenses and hearts tissue from diet groups before the start of the experiment (3 pooled samples in total).

Homogenization was performed to achieve the extraction of NAH and His for analysis. Each sample (lens, heart, or brain tissue) was weighted to the closest milligram and put in Eppendorf tubes with 600 µl ethanol (80% Et-OH) for homogenization. Afterward, the assay was centrifuged for 30 minutes at 1200 g in an Eppendorf centrifuge (Eppendorf AG, Germany). 500 µl assay medium was used for further analyses. The 500 µl of assay underwent further centrifugal treatment for 10 minutes at 12675 g. 200 µl of the aliquot of supernatant from the assay was extracted, and the dissolving agent was removed by evaporation at 40°C in an incubator (Termaks, Bergen, Norway). Following this, the assay was treated with a secondary dissolvement in 200 µl Na-Phosphate-Buffer (pH = 2.0), filtrated through a 22 µm syringe filtrate, and placed in a test tube which was inserted into a chromacol tube.

The assays were then placed onto a sample carousel and injected automatically by the Waters 717 Autosampler into the column (Zorbax SB250 x 4.6 mm ID, reversed-phase C18, 5 µm, Agilent Technologies AS, Norway). The eluents used as a solvent to separate the elution components were pumped in with the Waters 600 controller/pump module. First, 10 minutes eluent I with 0.6 ml min^{-1} flow (0,1 M phosphate buffer, pH 2.0). Following this was 10 minutes of eluent II with 1 ml min^{-1} (methanol: water, 1:1), ending with 25 minutes of column conditioning with eluent I. To quantify the absorbance at 210 nm, a Waters 486 Absorbance detector was utilized. The resulting data was integrated with Waters Empower software. An external standard curve was used to quantify assay concentration, with 0.25 mM NAH and 0.25 mM His standard for level 1 and 0.50 mM NAH and 0.50 mM His standard for level 2. Linear measurements were used in the method, including both NAH and His, between 0.08 µmol g^{-1} to 20 µmol g^{-1} when injecting 10 µl on the column. The limit of detection (LOD) is 0.08 µmol g^{-1}.

2.5.2 Free amino acid concentrations

The tissues were prepared to quantify free amino acid concentrations as described in section 2.5.1 and by methods described by (Breck et al., 2005).

Dissolvement of tissue was needed before a BioChrom 20 Plus Amino Acid Analyser (BioChrom Ltd, Cambridge, United Kingdom) based on low-pressure ion-exchange chromatography could be used. The samples were dissolved in running buffer (Lithium Citrate Loading Buffer, 80-2038-10, BioChrom Ltd, Cambridge, United Kingdom). Gradient elution systems were used to detect either complete free amino acid profiles or a shortened version – profiles of basic amino acids only. After performing amino acid analysis by low-pressure ion-exchange chromatography, post-column ninhydrin derivatization, colorimetric detection was performed at 570 and 440 nm (Waters 486, Waters Corporation).

2.7 Calculations and statistical analysis

2.7.1 calculations

Calculations performed were as followed;

1. Specific growth rate (SGR);

   Growth, as increase or decrease in a unit mass of individual fish, measured in the SI-unit gram, calculated using specific growth rate (SGR);

$$100\% \times \frac{lnw_2 - lnw_1}{d}$$

Where w2 indicates the final weight in SI-unit gram and w1 indicates the initial weight in SI-unit gram, d is a total number of experimental days (d = x 24h).

2. Condition factor (K);

$$\frac{w}{L^3} \times 100$$

Where w indicates weight measured in SI-unit gram (g), and L is fork length measured in SI-unit centimeters (cm).

3. Feed conversion Ratio (FCR)

Feed intake, as the ratio between feed employed divided by the increase in body mass, calculated using Feed conversion Ratio (FCR);

$$\frac{Feed\ employed\ (g)}{Increase\ in\ body\ mass\ (g)}$$

Where Feed employed represent feed consumed by fish, measured as a value with SI mass unit gram, and "Increase in body mass (g)" represents the value of "the weight at sampling (g)" subtracted from "initial weight (g), given in SI mass unit gram.

4. Digestible histidine in diets, calculated by using the macronutrient Apparent Digestibility Coefficients (ADC);

$$100 - 100(\left(\frac{\%Y_2O_3\ feed}{\%Y_2O_3\ faeces}\right) \times (\%nutrient\ faeces\ \times \%\ nutrient\ feed))$$

Where "%Y_2O_3 feed" represent the added yttrium in feed, "%Y_2O_3 faeces" represents the measured yttrium in faeces after employment, while "% nutrient feed" represents the known amount of nutrients in feed as a % of total feed composition, and "% nutrient faeces" represent the measured nutrients in faeces left over after employment as a % of total leftover in feed after employment.

5. Apparent dry matter digestibility (DMD) estimates were based on total collected faeces and calculated by the equation:

$$DMD = \left[\frac{DMI - DMF}{DMI}\right] * 100$$

Where DMI and DMF are a dry matter of feed and faeces, respectively.

2.7.2 Statistical analysis

Statistical analysis was conducted using the Statistica software (2021 StatSoft, Inc.). Values that are presented are given as means ± standard error (SEM). If not stated otherwise, all measurements were taken as tank samples from the trial populations (1 measurement per tank, resulting in 3 measurements per experimental diet). 95% Confidence interval (after this called CI) was used for all tests, giving a probability level of 5% ($p<0.05$), unless stated otherwise.

Levene tests were performed to check for the homogeneity of variances, and if non-significant ($P>0.05$), an equal variance was assumed. If the Levene test was significant, an equal variance was not assumed and was subjected to square root, log, or arcsin transformation before analysis. If the equal variance was not achieved by square root, log, or arscin transformation, a non-parametric Kruskal–Wallis H test or Mann-Whitney U test was performed. However, If $n > 30$, and $n_1 \sim n_2$, it is assumed that the data is robust toward violations of homogeneity of variance (Zar, 2010). Due to replicating tanks, nested analysis of variance (ANOVA) was performed using mean values of either tanks or individual samples nested in tanks to evaluate results obtained from different diets and tanks. Using the nested ANOVA, it is possible to control for any additional variation caused by tank-specific environmental factors or by hierarchical

feeding that may appear in fish feed trials (Ling & Cotter, 2003; Ruohonen, Vielma, & Grove, 1998). The effect of the His source, His level, and their interaction on feed intake, feed conversion ratio, fish performance, cataract development, tissue composition, and digestibility were analyzed by nested two-way ANOVA or two-way ANOVA where nesting was not possible. The effect of non-supplemented, medium and high supplementation from each His source on feed intake, feed conversion ratio, fish performance, cataract development, tissue composition, and digestibility were analyzed using one-way ANOVA. If either the one-way ANOVA or the two-way ANOVA resulted in $P<0.05$, a Tukey's honestly significance difference *posts hoc* test was performed. A Spearman rank coefficient of correlation was used to measure the correlation between weight and cataract score by measuring the direction of the association between the two ranked variables. Regression analysis was performed by using GraphPad Prism version 6.0 (2021 GraphPad Software, Inc.). Doing the regression analysis enabled testing whether a linear or a non-linear relationship existed between His supplementation level and cataract score and tissue concentrations. The best-fit regression lines are shown in the figures, indicated by R^2 value, and the best-fit equation is given in their legends. The dotted lines represent 95% CI.

3. Results

3.1 Feed and feed efficiency

3.1.1 Feed composition

Proximate composition and feed formulation were achieved as planned, reflecting an up-to-date industry recipe (*Tables 1 and 2*). The amino acid composition for His did diverge somewhat from the planned target levels. The non-supplemented control diet had a target level of 0.80%, and the feed analysis showed 0.93% His. All medium diets had a His level on par with their target level of 1.2% His. The high diets diverged from their target level of 1.4% His. AH and CH had 1.3% His while KH had 1.5% His.

3.1.2 Feed intake

Feed intake (FI) was steady for all groups for both experimental and control throughout the feed trial. On balance, feed intake was lowest during the first week after seawater transfer, followed by a steady increase in FI over the next five weeks.

The mean FI for experimental groups during the trial was 6065g (*Table 3*, SEM 134g, n = 3 per diet, range 4752 – 6965g). No difference was found in FI for fish fed the experimental diets (two-way ANOVA, $P>0.05$, n = 3 per diet). No difference was found when comparing fish fed the CTRL diet with fish fed the medium and high diets of each His source (one-way ANOVA, $P>0.05$, n = 3 per diet).

3.1.2 Feed conversion ratio (FCR)

The mean FCR for the experimental groups was 0.84 (SEM 0.04, n = 3 per diet, range 0.69-1.26). No difference was found in the FCR for the experimental groups (two-way ANOVA, $P>0.05$, n = 3 per diet). Fish given the AM and KH diets had a higher FCR than CTRL (one-way ANOVA, $P<0.05$, n = 3 per diet).

3.1.3 Digestibility

The digestibility did not vary between the control feed or the experimental feed. The mean ADC for histidine was 90.44% (SD 1.64, n = 3 per diet), and increasing with increasing histidine supplementation.

3.2 Key fish performance metrics

3.2.1 Growth

The Atlantic salmon achieved on average slightly below two-fold weight increase during the feed trial. Following termination of the feed trial, the mean weight of the individual fish was recorded at 308g (SEM 2g, n = 1248, range 84 - 549g).

Lower final weight was identified for fish given the CM diet versus the CTRL diet, while fish fed the KM diet achieved higher final weight than the CTRL diet (one-way ANOVA, $P<0.05$). Furthermore, a lower final weight was identified for the fish given the KH- and CM-diets versus the KM-diet (*Table 3*, nested ANOVA, $P<0.05$, n = 1066). In addition, fish provided the CM-diet achieved lower final weight than AM- and AH-diets (*Table 3*, nested ANOVA, $P<0.05$, n = 1066). Fish provided with diets supplemented with His from the C-source resulted in lower final weight diets supplemented with His from the A-source (nested ANOVA, $P>0.05$, n = 1066).

3.2.2 Specific growth rate (SGR)

The daily growth rate (*Table 3*), measured as SGR, was 1.26 (SEM 0.06, n = 3 per diet, range 0.79 – 1.59). Fish provided with the experimental diets had SGR similar to CTRL (one-way ANOVA, P>0.05, n = 3 per diet), except for the AH diet, which achieved a lower SGR (one-way ANOVA, P<0.05). The source and level of dietary His did not influence the growth rate (*Table 3*, two-way ANOVA, $P>0.05$, n = 3 per diet).

3.2.3 Condition factor (K)

Condition factor increased during the trial period (*Table 3*), and the mean K at trial termination was 1.26 (SEM 0.05, n = 1248, range 0.32 – 4.07). No difference was found for fish fed CTRL diets versus fish fed experimental diets (one-way ANOVA, *P>0.05*). No differences could be identified between experimental groups in a two-way ANOVA (*Table 3*).

3.2.4 Organ indexes (Viscera-somatic index – VSI (%), Hepato-somatic index – HIS (%), and Cardio-somatic index CSI (%)

The key organ indexes of fish provided with the CTRL diets achieved similar organ indexes as the fish given experimental diets (one-way ANOVA, *P>0.05*). Fish given the medium-level his diets gained higher KOIs than fish fed the high-level His diets (nested ANOVA, *P<0.05*, n = 108). No differences were found in KOIs for fish given different His sources (*Table 3*, nested ANOVA, *P>0.05*, n = 108).

Table 3. Somatic data from triplicate groups of post-smolt Atlantic salmon fed three levels of dietary His for 6 weeks.
(Mean values with their standard errors) H.S = Histidine source, H.L = Histidine level, (H.S x H.L) = Experimental group.

	Dietary Groups									
	CTRL	AM	AH	CM	CH	KM	KH	Statistic†		
Growth and utilization								H.S	H.L	(H.S x H.L)
SGR	1.47 (0.07)	1.41 (0.06)	1.36 (0.05)	0.96 (0.15)	1.32 (0.17)	1.46 (0.07)	1.05 (0.04)	n.s	n.s	n.s
FCR	0.72 (0.01)	0.82 (0.02)	0.77 (0.01)	1.05 (0.13)	0.82 (0.11)	0.74 (0.04)	0.90 (0.06)	n.s	n.s	n.s
Start Weight (g)	167 (9)	172 (7)	176 (5)	191 (11)	176 (11)	172 (9)	192 (14)	n.s	n.s	n.s
Final Weight (g)	309 (14)	312 (11)^{a}	313 (11)^{a}	286 (15)^{b}	307 (10)^{b}	318 (14)^{ab}	298 (8)^{ab}	p<0.05	n.s	p<0.05
Weight Gain (g)	142 (5)	140 (10)	136 (8)	95 (17)	131 (17)	146 (10)	106 (2)	n.s	n.s	n.s
Final K	1.26 (0.02)	1.25 (0.02)	1.29 (0.02)	1.25 (0.02)	1.26 (0.02)	1.26 (0.01)	1.25 (0.00)	n.s	n.s	n.s
Final CSI (%)	0.12 (0.01)	0.12 (0.01)^{a}	0.11 (0.01)^{b}	0.13 (0.00)^{a}	0.11 (0.00)^{b}	0.13 (0.00)^{a}	0.12 (0.00)^{b}	n.s	p<0.05	n.s
Final VSI (%)	7.48 (0.06)	7.86 (0.41)^{a}	6.66 (0.59)^{b}	7.76 (0.24)^{a}	6.49 (0.36)^{b}	7.59 (0.41)^{a}	7.12 (0.32)^{b}	n.s	p<0.05	n.s
Final HSI (%)	1.18 (0.05)	1.23 (0.07)^{a}	1.14 (0.09)^{b}	1.27 (0.04)^{a}	1.09 (0.04)^{b}	1.3 (0.08)^{a}	1.07 (0.05)^{b}	n.s	p<0.05	n.s
Cataract Score	2.6 (0.7)	2.4 (0.4)^{ac}	2.0 (0.2)^{abc}	1.8 (0.4)^{b}	2.2 (0.5)^{abc}	2.3 (0.5)^{ac}	1.8 (0.1)^{b}	n.s	n.s	p<0.05
FI (g)	6256 (724)	6333 (593)	6266 (724)	5586 (794)	6096 (154)	6380 (689)	5545 (400)	n.s	n.s	n.s
FI (g) per fish	103 (12)	111 (13)	104 (12)	95 (15)	104 (4)	106 (13)	95 (8)	n.s	n.s	n.s

AM - 1.2%His; AH - 1.4%His; CM - 1.2%His; CH - 1.4%His; KM - 1.2%His; KH - 1.4%His.
† Final weight, Final K, CSI (%), HSI (%), VSI (%) and cataract score were analysed using nested two-way ANOVA and Tukey's honestly significant difference post hoc test. FCR, Start weight (g), weight gain (g), FI (g) and FI (g) per fish were analyzed using two-way ANOVA and Tukey's honestly significant difference post hoc test. (NS: P>0.05). If Levenes test *P<0.05* , non-parametric tests were used. $^{a\,b\,c}$ Mean values with unlike letters were significant (P<0.05).

3.3 Cataract prevalence and severity

3.3.1. Feed trial initiation

A mean cataract prevalence of 58% and a mean cataract score of 0.6 (n = 64) were recorded at the start of the feeding trial.

3.3.2 Feed trial termination

The mean prevalence of cataract at the trial termination was 96.4%. All experimental feed groups and CTRL indicated elevated cataract severity from initiation to trial termination (*Fig 2*). Cataract severity was reduced in all groups compared to CTRL except the fish given the AM-diet (one-way ANOVA, $P<0.05$, n = 90 per diet), and fish given the CM diet achieved lower cataract score than CH diet (one-way ANOVA, $P<0.05$, n = 90 per diet). Cataract severity was lower in fish given the CM and KH diets than AM and KM (nested ANOVA, $P<0.05$, n = 90 per diet).

A Spearman's rank correlation was performed with final cataract score versus weight for experimental groups on a tank level, and a non-significant correlation (R = 0.06) was identified between the two values ($P>0.05$, n = 630).

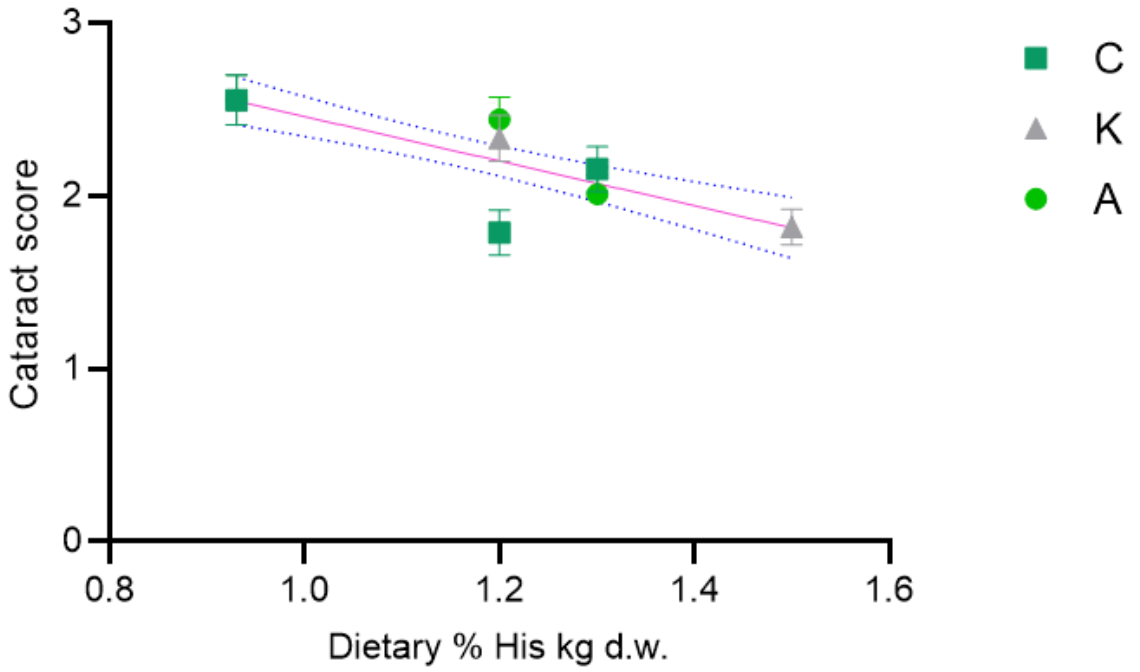

Fig. 3. Cataract score at the trial termination in relation to the analyzed His levels in diets given the A-source , C-source , and K-source His at medium and high level. CTRL was included as the start point for all three regression analyses. Values are means with their standard error represented by vertical bars. 95% CI is represented by dotted lines. The His source did not affect the cataract score, and the results for all three His sources could be expressed as the same equation: -1.29x + 3.75 (R^2 0.04).

The regression analysis identified a negative linear relationship between cataract score and His supplementation level at the trial termination (*Fig 3*) and that the cataract severity had a negative relationship with His supplementation levels (R^2 = 0.04). The His source did not affect the cataract score, and a common straight line could express the difference in cataract score between the His sources at medium and high supplementation levels: Cataract score = -1.29x + 3.75 (R^2 0.04); where x represents the His supplementation level.

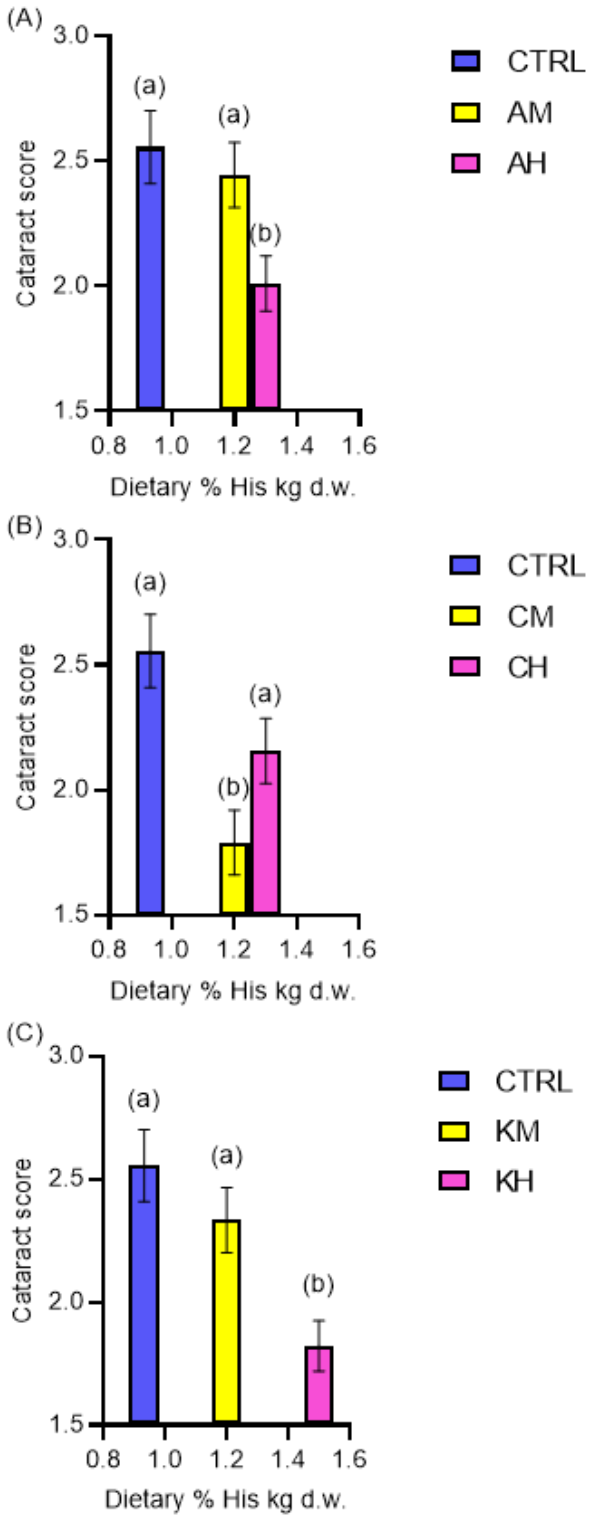

Fig. 2. Cataract score at the feed trial termination in relation to analyzed levels of His in the diet for CTRL, and the A-source (A), the C-source (B), and the K-source (C) at a medium and high level. Values are means with their standard error represented by vertical bars. [a, b] Mean values with different letters were significantly different when comparing CTRL to experimental groups at medium and high His level (one-way ANOVA, *P<0.05*).

3.4 Lens Tissue

3.4.1 Lens free histidine

Before the trial initiation, lens His was similar between all experimental groups with a mean value of 0.95 µmol/g His (SEM 0.14 µmol/g, n = 3, range 0.72 – 1.20 µmol/g).

The lens His concentration was higher in fish given the high supplementation diets compared to the medium diets (two-way ANOVA, $P<0.05$, n = 3 per diet) and the control (*Fig 4*, one-way ANOVA, $P<0.05$, n = 3 per diet). No differences were seen between His sources (two-way ANOVA, $P>0.05$, n = 3 per diet).

The regression analysis identified a positive linear relationship ($R^2 = 0.61$) between lens free His and His supplementation level at the trial termination (*Fig 5A*), where the lens free His had a positive relationship with His supplementation levels. The His source did not affect the lens free His, and the results for all three His sources could be expressed as the same equation of a common straight line: Lens free His = 1.63x -0.48 ($R^2 = 0.61$); where x represents the His supplementation level.

3.4.2 Lens N-acetyl histidine

NAH was similar at the feed trial initiation between all experimental groups with a mean value of 5.00 µmol/g (SEM 0.31 µmol/g, n = 3, range 4.40 – 5.40 µmol/g).

The lens NAH concentration was higher in all fish groups given the His supplemented diets than the control group (*Fig 4*, one-way ANOVA, $P<0.05$, n = 3 per diet), and the fish given the KH- and CH-diets had higher lens NAH concentrations compared to their respective medium levels (two-way ANOVA, $P<0.05$, n = 3 per diet), while no difference was seen between AH and AM (two-way ANOVA, $P>0.05$, n = 3 per diet).

A regression analysis identified a linear relationship between NAH and His supplementation level at the trial termination (*Fig 5B*), where the lens NAH had a positive relationship with His supplementation levels (R^2=0.60). The His source did not affect the lens free NAH, and the results for all three His sources could be expressed as the same equation for a straight line: Lens NAH = 1.63x – 0.48 ($R^2 = 0.60$); where x represents the His supplementation level.

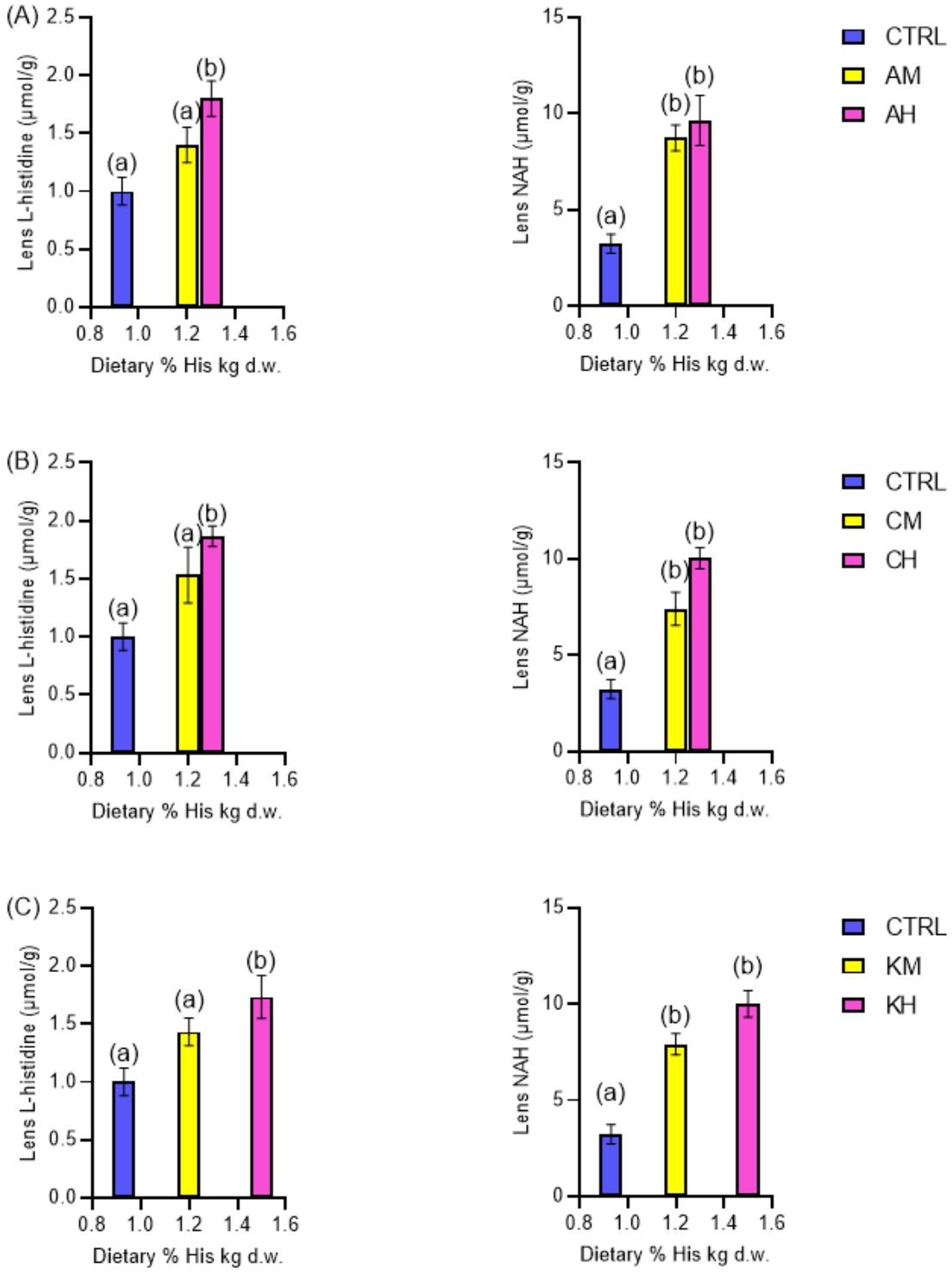

Fig. 4. Lens His and lens NAH at the trial termination for the control group compared to experimental groups given the A-source , C-source , and K-source His at medium and high level. Values are means, with their standard error represented by vertical bars. [a, b] Mean values with unlike letters were significantly different when comparing CTRL to experimental groups at medium and high His level (one-way ANOVA, *P<0.05*).

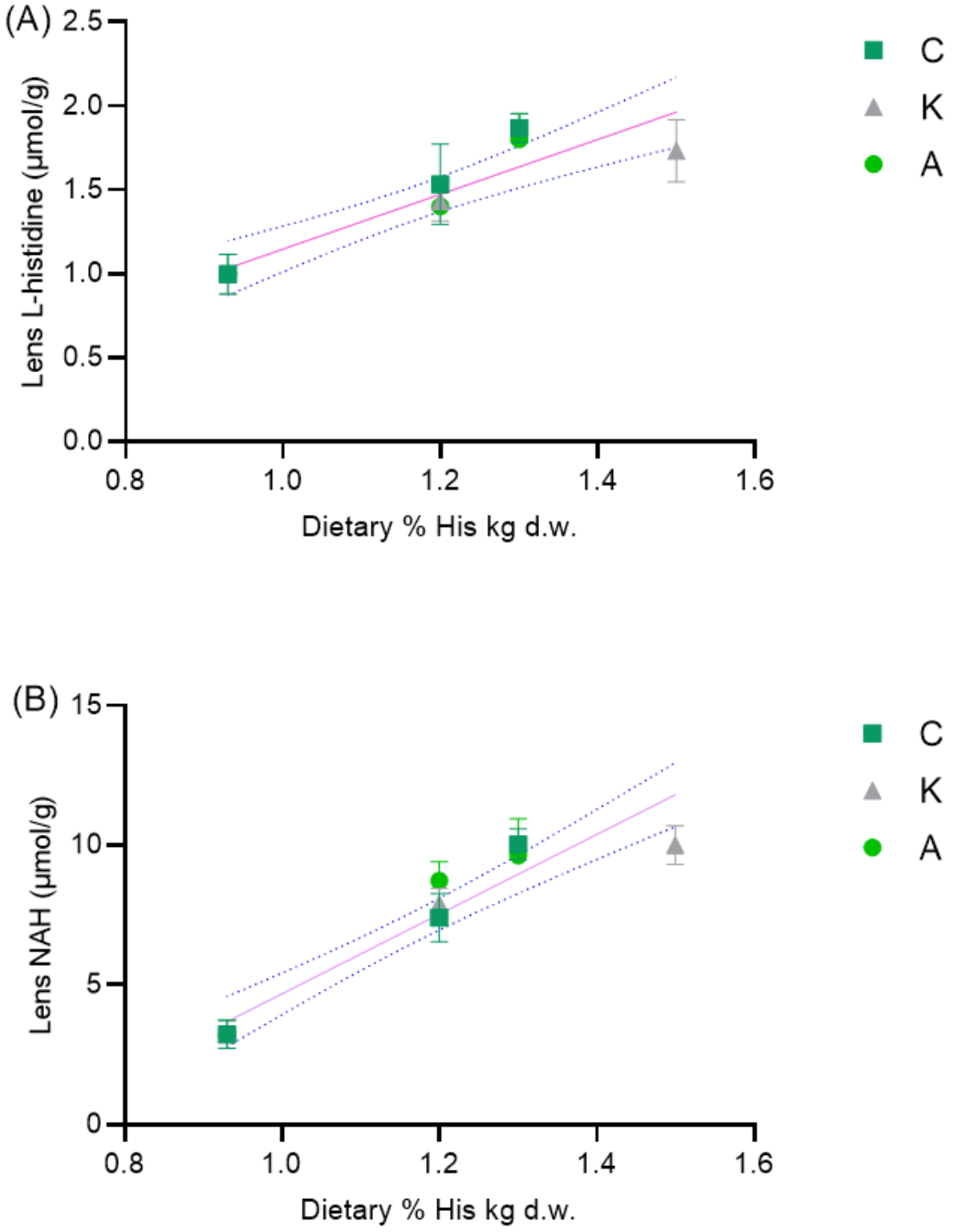

Fig. 5. Lens His (A) and NAH (B) at the trial termination in relation to the analyzed His levels in diets given the A-source , C-source , and K-source His at medium and high level. CTRL was included as the start point for all three regressions analyses. Values are means with their standard error represented by vertical bars. 95% CI is represented by dotted lines. The His source did not affect the lens His (A) or Lens NAH (B), and the results for all three His sources could be expressed as the same equation: (A) 1.63x – 0.48 (R^2 0.60) and (B) 14.25x – 9.56 (R^2 0.80).

3.5 Heart Tissue

3.5.1 Heart free histidine

At the feed trial initiation, heart free His was similar between all groups with a mean value of 0.41 µmol/g (SEM 0.03 µmol/g, n = 3, range 0.36 – 0.47 µmol/g).

The heart His (*Fig 6*) was higher in fish fed high His diets compared to medium His diets (two-way ANOVA P<0.05, n = 3 per diet). No difference in Heart His was found between His sources (two-way ANOVA, P<0.05, n = 3 per diet). The heart His was not different in fish fed CTRL compared to his supplemented diets (*Fig 6*, One-way ANOVA, *P<0.05*), except for fish provided the KH-diet, which had a higher concentration of heart His compared to CTRL (one-way ANOVA, P<0.05, n = 3 per diet).

The regression analysis shown in *Fig 7A* identified a positive linear relationship between heart free His and His supplementation level where the heart free His had a positive relationship with His supplementation levels (R^2 = 0.60). the His source did not affect the heart free His, and the results for all three His sources could be expressed as the same equation for a straight line: Heart free His = 0.98x – 0.41 (R^2 = 0.60); where x represents the His supplementation level.

3.5.2 Heart NAH

At the feed trial initiation, heart NAH was similar between all groups with a mean value of 2.77 µmol/g (SEM 0.15 µmol/g, n = 3, range 2.5 – 3.0 µmol/g). The heart NAH was not different for fish fed CTRL compared to experimental diets (*Fig. 6*, one-way ANOVA, *P>0.05*, n = 3 per diet), except for fish provided CH- and KH-diets which achieved higher heart NAH concentration than fish fed CTRL-diets (one-way ANOVA, *P<0.05*, n = 3 per diet). The heart NAH concentration was higher in fish groups given the high supplementation diets versus the medium diets (two-way ANOVA, *P<0.05*, 3 per diet). No difference was found in heart NAH concentrations between His sources (Kruskal–Wallis H test, *P>0.05*, n = 3 per diet).

The regression analysis in *Fig. 7B* identified a positive linear relationship between heart NAH and His supplementation level where the heart NAH had a positive relationship with His supplementation levels (R^2 = 0.46). The His source did not affect the heart NAH, and the results

for all three His sources could be expressed as the same equation for a straight line: Heart NAH = 1.24x + 1.51 (R^2 = 0.46); x represents the His supplementation level.

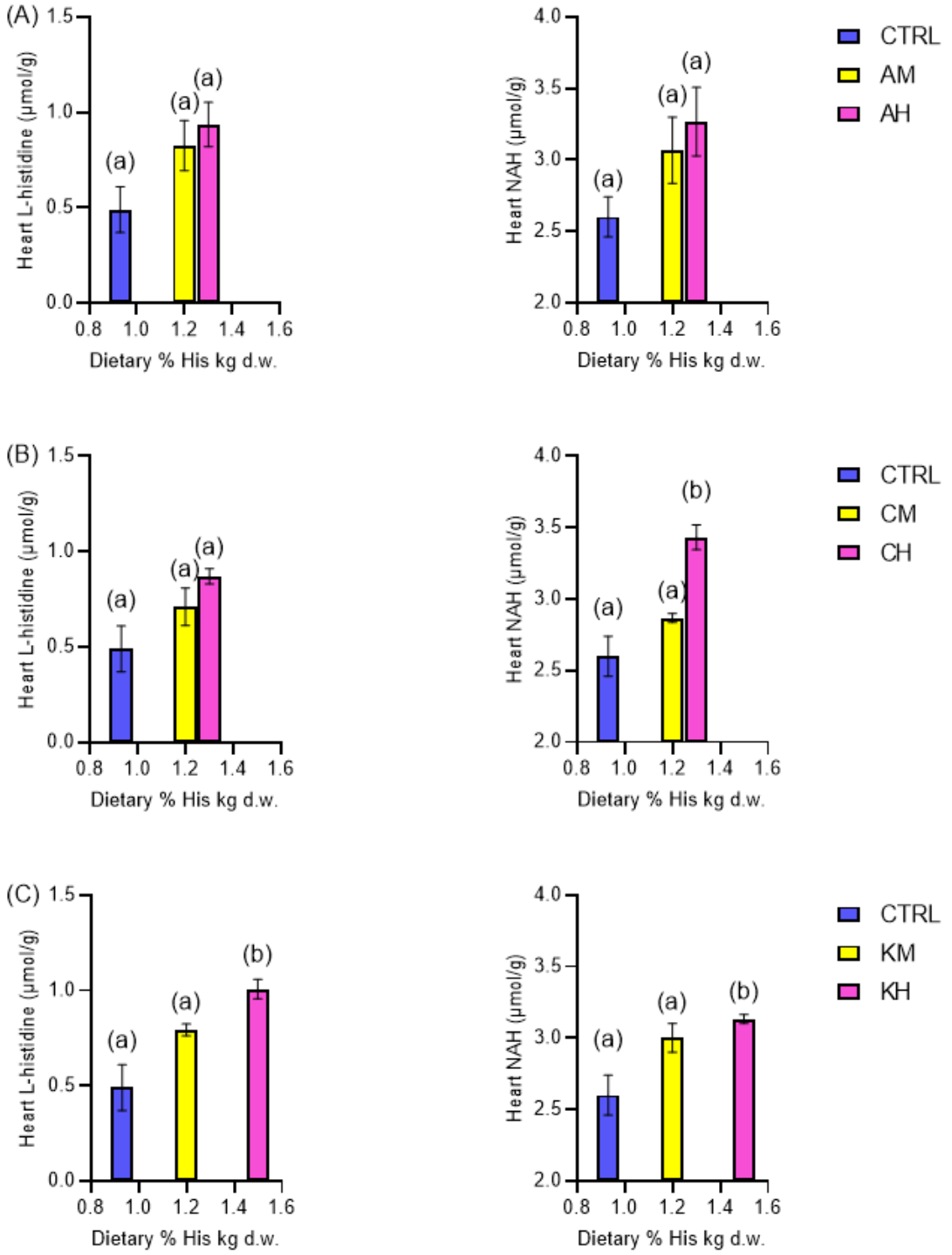

Fig. 6 Heart His and Heart NAH at the trial termination for the control group compared to experimental groups given the A-source (A), C-source (B), and K-source (C) His at medium and high level. Values are means, with their standard error represented by vertical bars. [a, b] Mean values with unlike letters were significantly different when comparing CTRL to experimental groups at medium and high His level (one-way ANOVA, *P<0.05*).

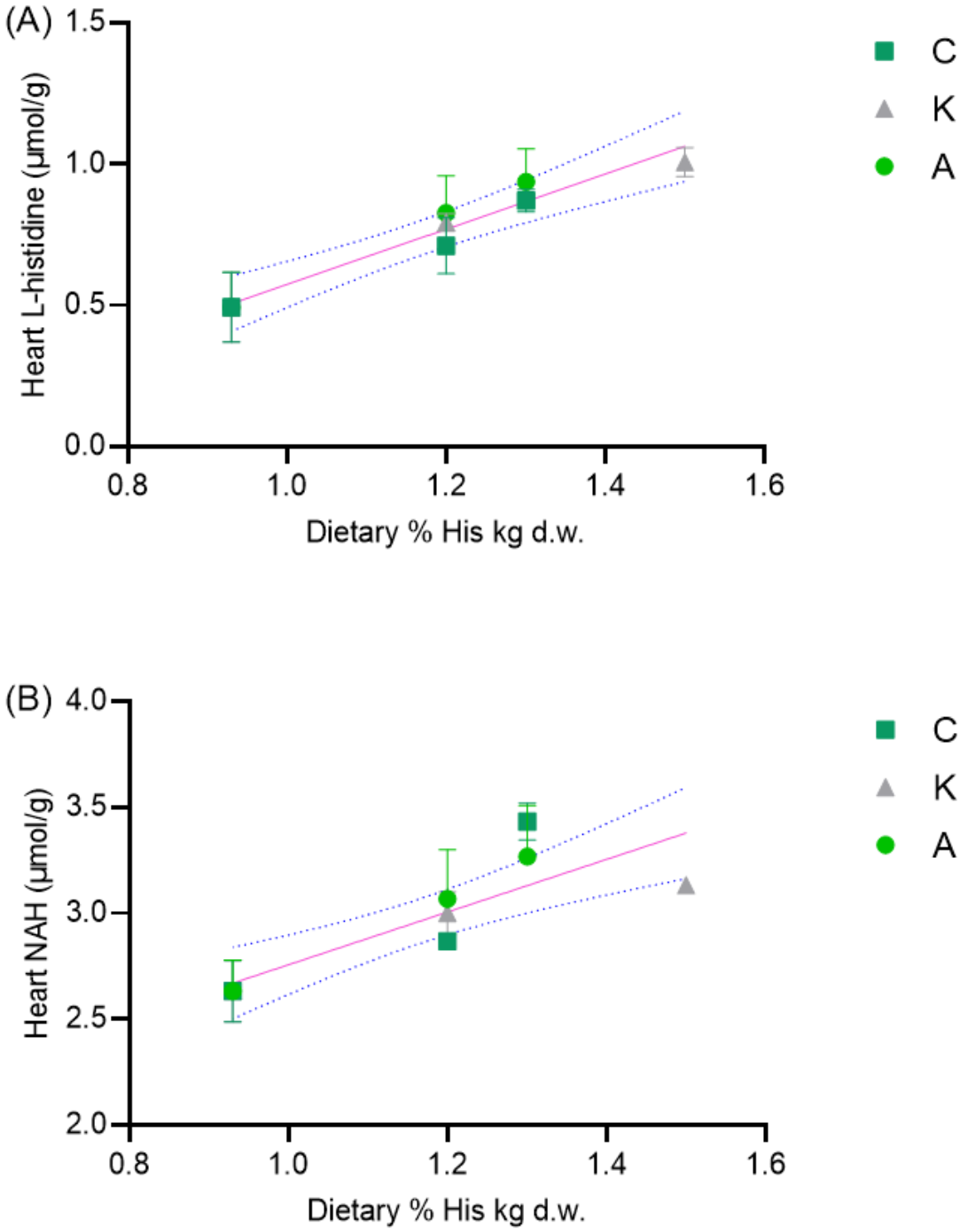

Fig. 7. Heart His (A) and NAH (B) at the trial termination in relation to the analyzed His levels in fish given diets with the A-source , C-source , and K-source His at medium and high level. CTRL was included as the start point for all three regressions analyses. Values are means with their standard error represented by vertical bars. 95% CI is represented by dotted lines. The His source did not affect the heart His (A) or heart NAH (B) concentrations, and the results for all three His sources could be expressed as the same equation: heart His (A) 0.98x - 0.41 (R^2 0.60) and heart NAH (B) 1.24x + 1.51 (R^2 0.46).

3.6 White muscle tissue

3.6.1 White muscle free histidine

The mean muscle concentration of free His at the trial initiation was 0.19 µmol/g (SEM 0.04 µmol/g, n = 3, range 0.14 – 0.26 µmol/g). No differences were found when comparing CTRL to medium and high diets of each His source, except for fish given the CH and KH diets which had higher muscle concentration of free His than CTRL (*Fig 8*, one-way ANOVA, $P<0.05$, n = 3). Similar His levels in fish muscle tissue given the experimental diets were found, but fish given the high His diets had higher levels than fish given the medium diets (two-way ANOVA, $P<0.05$, n = 3).

The regression analysis identified a positive linear relationship between white muscle free His and His supplementation level at the trial termination (*Fig 9A*), where white muscle free His had a positive relationship with the His supplementation level (R^2 0.70). The His source did not affect the white muscle free His, and a common straight line could express the difference in white muscle free His between the His sources at medium and high supplementation levels: White muscle free His = 2.54x – 2.21 (R^2 0.70); where x represents the His supplementation level.

3.6.2 White muscle anserine

At the trial initiation, the mean muscle concentration of anserine was 17.08 µmol/g (SEM 0.22 µmol/g, n = 3, range 16.85 – 17.53 µmol/g). Fish given the experimental diets, including both medium and high levels of His supplementation, had higher muscle tissue anserine levels than fish given the CTRL diet (*Fig 8*, one-way ANOVA, $P<0.05$, n = 3 per diet). No difference was found between experimental diets, except for His level, where fish given the high His diets had higher anserine level than fish given the medium His diets (two-way ANOVA, $P<0.05$, n = 3).

The regression analysis identified a non-linear relationship between white muscle anserine and His supplementation level at the trial termination (*Fig 9B*), where white muscle anserine had a positive relationship with His supplementation levels ($R^2 = 0.96$). The His source did not affect the white muscle anserine levels, and the concentration in white muscle anserine between the His sources at medium and high supplementation levels could be expressed by a second-degree polynomial regression, describing the following relationship: White muscle anserine = $-20.69x^2 + 55.91x - 19.88$ ($R^2 = 0.96$); where x represents the His supplementation level.

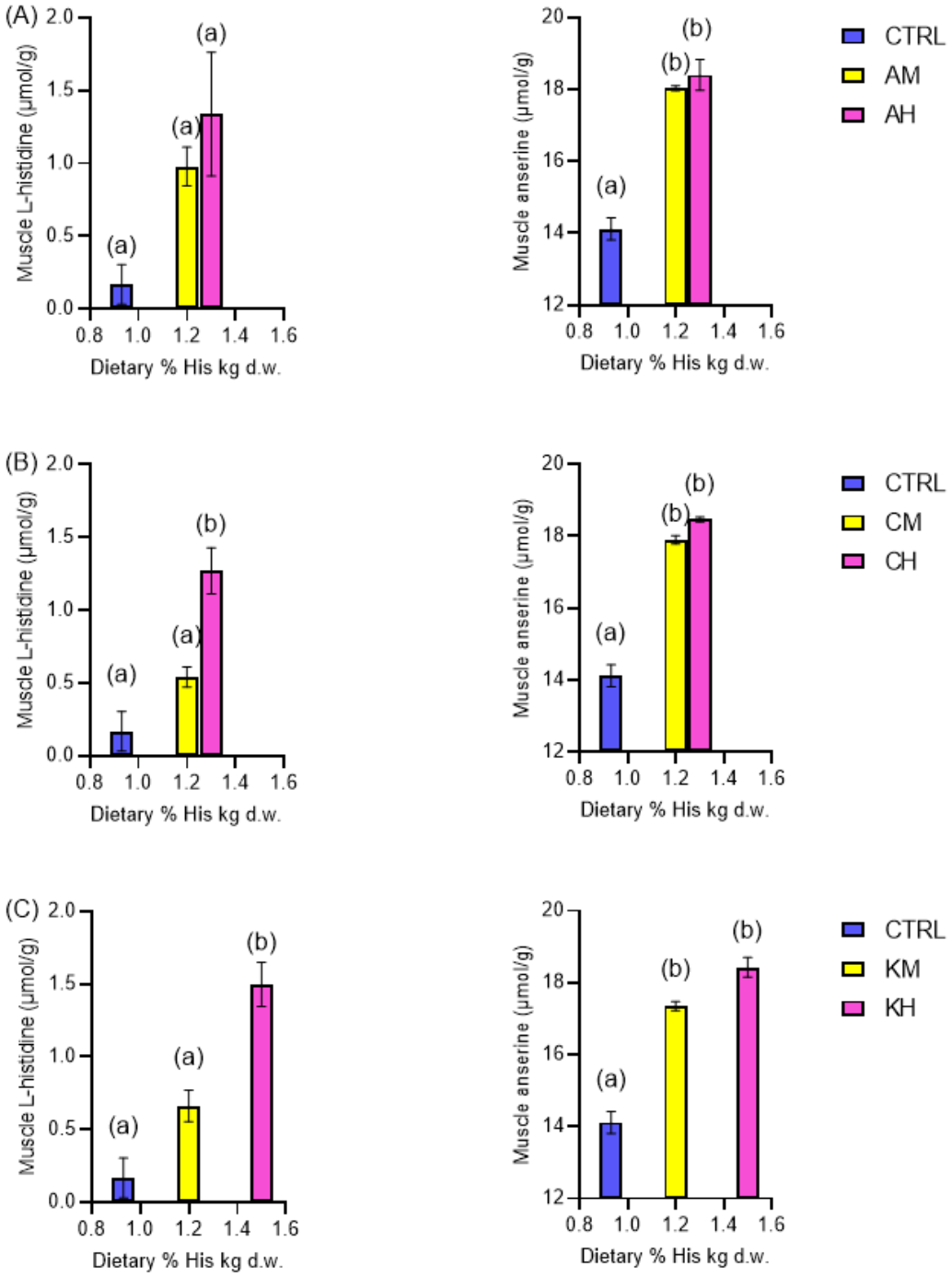

Fig. 8. His and Anserine in white muscle at the trial termination for the control group compared to experimental groups given the A-source , C-source , and K-source His at medium and high level. Values are means, with their standard error represented by vertical bars. [a, b] Mean values with unlike letters were significantly different when comparing CTRL to experimental groups at medium and high His level (one-way ANOVA, *P<0.05*

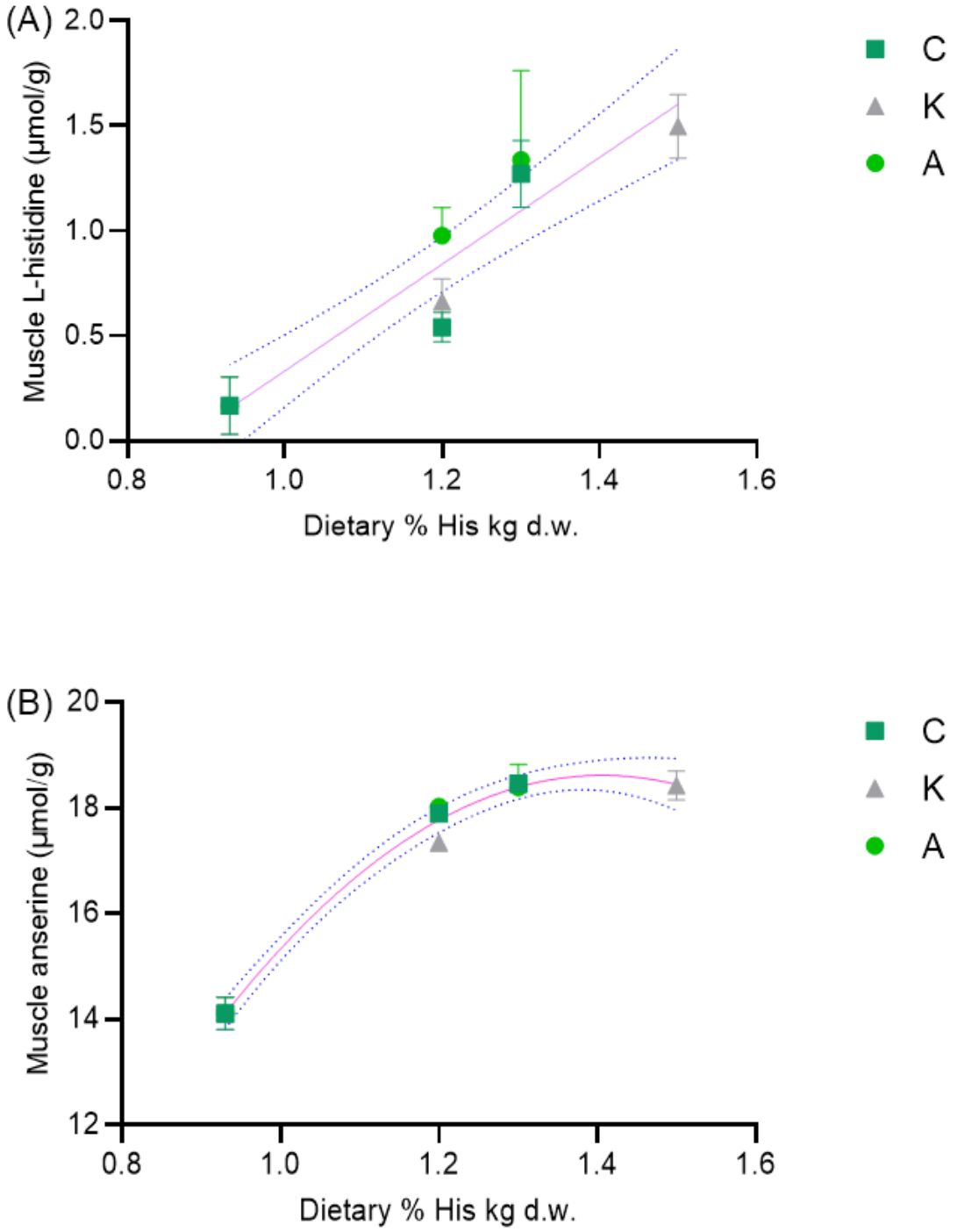

Fig. 9. His (A) and anserine (B) in white muscle at the trial termination in relation to the analyzed His levels in diets with the A-source , C-source , and K-source His at medium and high level. CTRL was included as the start point for all three regressions analyses. Values are means with their standard error represented by vertical bars. 95% CI is represented by dotted lines. The His source did not affect the white muscle His (A) or muscle anserine (B), and the results for all three His sources could be expressed as the same equation: white muscle His (A) 2.54x – 2.21 (R^2 0.70) and muscle anserine (B) $-20.69x^2 + 55.91x – 19.88$ (R^2 0.96).

3.6.3 White muscle carnosine

The mean muscle concentration of carnosine was 0.56 µmol/g (SEM 0.10 µmol/g, n = 3, range 0.45 – 0.76 µmol/g) at the trial initiation. Except for fish given the AM diet, all experimental diets had higher muscle tissue carnosine levels than fish given the CTRL diet (*Fig 10*, one-way ANOVA, $P<0.05$, n = 3 per diet). No differences were found between experimental diets (two-way ANOVA, $P>0.05$, n = 3 per diet).

The regression analysis identified a linear relationship between white muscle carnosine and His supplementation level at the trial termination and that the white muscle carnosine had a positive relationship with His supplementation levels (*Fig 11A*). The His source did not affect carnosine, and a common straight line could express the difference in white muscle carnosine between the His sources at medium and high supplementation levels: White muscle carnosine = 1.50x + 1.22 ($R^2 = 0.74$), where x represents the His supplementation level.

3.6.4 White muscle β-Alanine

The mean muscle concentration of β-Alanine was 0.39 µmol/g (SEM 0.06 µmol/g, n = 3, range 0.29 – 0.51 µmol/g) at the trial initiation. Fish given the experimental diets had lower β-Alanine compared to fish given the CTRL diet (*Fig 10*, one-way ANOVA, $P<0.05$, n = 3). No difference was found for β-Alanine when comparing the His sources (Kruskal–Wallis H test, $P>0.05$, n = 3 per diet), but the medium His diets achieved a higher β-Alanine level than high His diets (Kruskal–Wallis H test, $P<0.05$, n = 3 per diet). No difference was found between the experimental groups except for KM, which had higher β-Alanine concentration than all other experimental diets except for CM (two-way ANOVA, $P<0.05$, n = 3).

The regression analysis identified a non-linear relationship between white muscle β-Alanine and His supplementation level at the trial termination, where white muscle β-Alanine had a negative relationship with His supplementation levels (*Fig 11B*). The His source did not affect white muscle β-Alanine, and a second-degree polynomial regression could express the relation between white muscle β-Alanine concentration and the His sources at medium and high supplementation levels: White muscle β-Alanine = $5.87x^2 – 7.51 – 2.57$ ($R^2 = 0.89$), where x represents the His supplementation level.

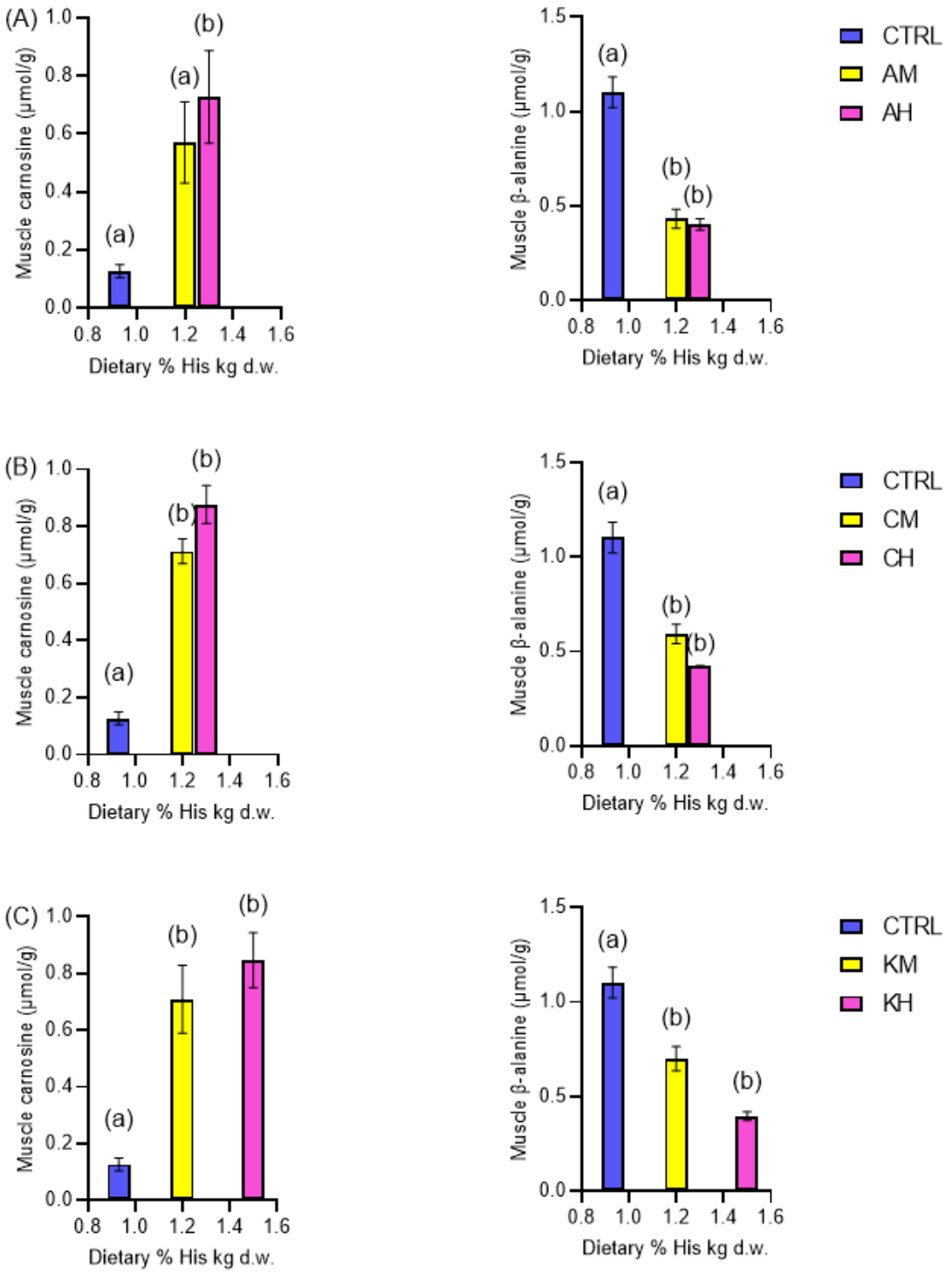

Fig. 10. Carnosine and β-Alanine in white muscle at the trial termination for the control group compared to experimental groups given the A-source , C-source , and K-source His at medium and high level. Values are means, with their standard error represented by vertical bars. [a, b] Mean values with unlike letters were significantly different when comparing CTRL to experimental groups at medium and high His level (one-way ANOVA, *P<0.05*)

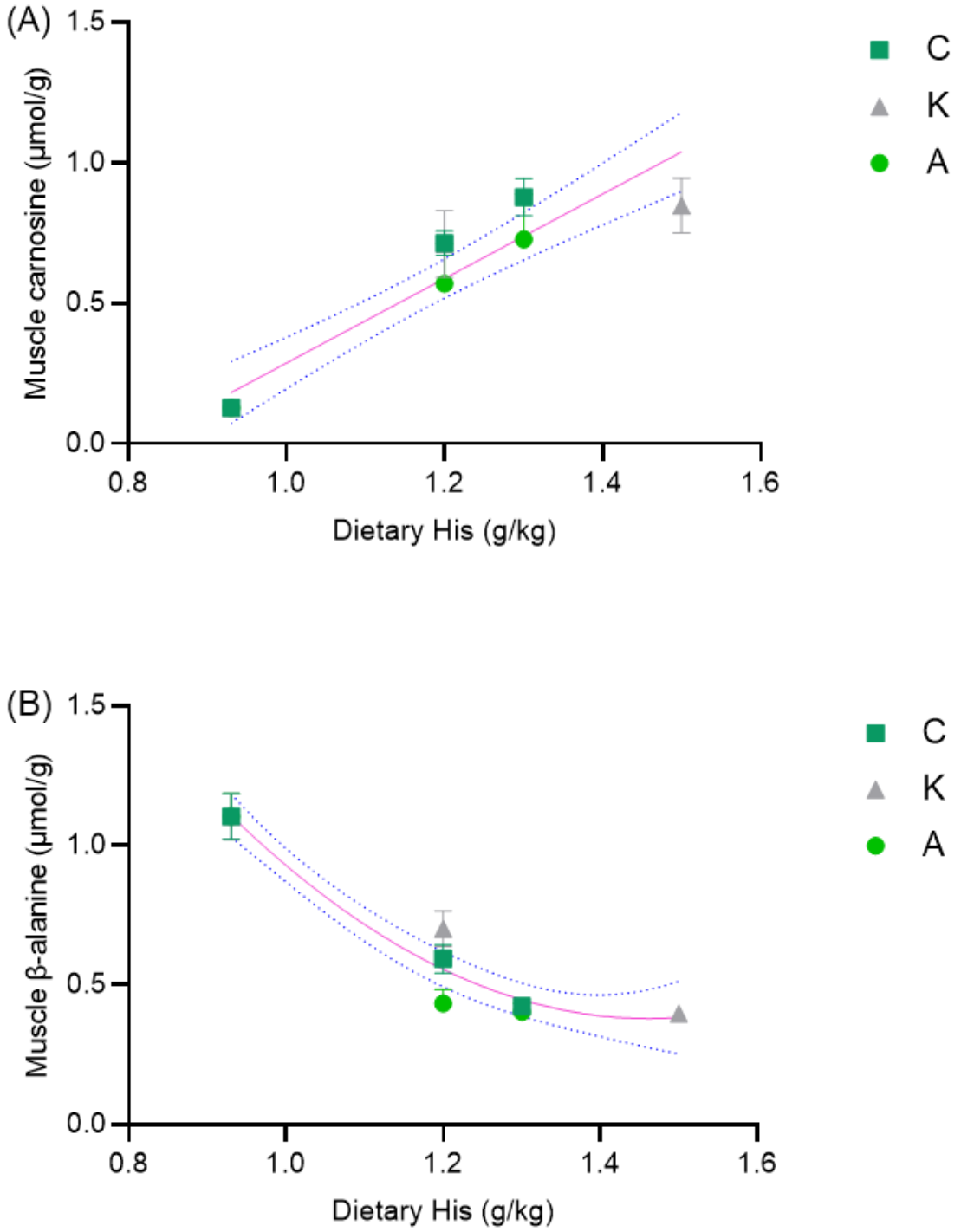

Fig. 11. Carnosine (A) and β-alanine (B) in white muscle at the trial termination in relation to the analyzed His levels in diets with the A-source , C-source , and K-source His at medium and high level. CTRL was included as the start point for all three regressions analyses. Values are means with their standard error represented by vertical bars. 95% CI is represented by dotted lines. The His source did not affect the muscle Carnosine (A) or β-alanine (B), and the results for all three His sources could be expressed as the same equation: muscle carnosine (A) 1.50x + 1.22 (R^2 0.74) and muscle β-alanine (B) $5.87x^2 – 7.51 – 2.57$ (R^2 0.89).

3.6.5 1-Methyl-L-Histidine

The mean 1-Methyl-L-Histidine concentration in muscle tissue was 0.017 µmol/g (SEM 0.003 µmol/g, n = 3, range 0.010 – 0.020 µmol/g) at the trial initiation. No differences were found for 1-Methyl-L-Histidine between fish given experimental diets (two-way ANOVA, $P>0.05$, n = 3 per diet) or when comparing them to fish given the CTRL diet (one-way ANOVA, $P>0.05$, n = 3 per diet). The mean 1-Methyl-L-Histidine concentration in muscle tissue at the trial termination was 0.021 µmol/g (SEM 0.001 µmol/g, n = 3 per diet, range 0.010 – 0.030 µmol/g).

4. Discussion

The study aimed to investigate the efficacy of new sources of L-Histidine monohydrochloride monohydrate to support tissue His status and cataract mitigation in Atlantic salmon smolt after seawater transfer by using up-to-date industrial diets.

All three suppliers of synthetic His in a formulated extruded pellet for salmon achieved similar feed utilization, fish performance, cataract development, and tissue levels of His and its metabolites; on par with findings from previous salmon nutritional studies (Remø et al., 2014; Sambraus et al., 2017; Waagbø et al., 2010). Within each supplier of synthetic His, the results varied according to dietary level, and especially for cataract and tissue levels of His and its metabolites, which underscore the importance of the relationship between cataract development and tissue concentrations of His and its metabolites with dietary His levels.

4.1 The histidine sources supported similar feed utilization and fish performance

A scientific evaluation of the efficacy of feed supplements requires a sound experimental design and a well conducted feeding study. The present study evaluated feed efficiency, growth, and somatic performance in relative organ indexes. No difference in feed intake and digestibility was found for any experimental group or CTRL. These findings are similar to other nutritional studies concentrating on His, where supplementation levels did not affect feed intake in smolt and postsmolt Atlantic salmon (Remø et al., 2014; Waagbø et al., 2010).

The CTRL diet did achieve similar FCR to the experimental diets except for AM and KH, which achieved higher FCR than the CTRL diets. No differences were found when comparing supplemented diets. The FCR in this study was similar to other studies on post-smolt Atlantic salmon (Hevrøy et al., 2015). After transfer to seawater, reduction in feed intake has been identified in previous studies (Handeland et al., 2003) and is assumed to be due to the metabolic adjustments related to smoltification (Clarke et al., 1981; Sheridan, 1989).

The Atlantic salmon grew slightly below two-fold during the feed trial. Fish in the CM and KH groups had the highest weight (on a biomass level) at the initiation of the feed trial yet ended with the lowest average weight (both on an individual and a biomass level) (*Table* 3). The weight differences within the CM and KH groups did not diverge more than slightly above one

standard deviation from the mean weight at initiation and thus deemed not to affect the results. Fish fed the non-supplemented CTRL diet achieved somatic growth similar to experimental diets, which is analogous with results from previous nutritional studies, where varying levels of His supplementation did not affect growth (Remø et al., 2014; Waagbø et al., 2010). Fish given the AM, AH, and KM diets achieved higher somatic weight than fish given the CM diets (*Table* 3). Fish fed the KH diet achieved a lower somatic weight than the KM diet. Higher final weight was found for fish given the A-source His supplementation than in C-source His supplementation, but no difference was found for His level. No underperformance was seen in other medium His diets. The fish given CM diet achieved the lowest SGR among the experimental groups and was lower than AM (*Table 3*). The AH group was the closest in absolute number to CM and had a lower SGR versus the CTRL group. No other experimental group had fish with lower or higher SGR versus the CTRL group. These findings are assumed to be driven by other factors than nutrition, due to their irregularity within His supplementation levels, and between His sources.

Previous studies have not found any correlation between His supplementation above the minimum requirement for growth and SGR (Remø et al., 2014; Waagbø et al., 2010). Breck et al. (2005) suggested that increased His supplementation through the feed to salmon smolt had growth-enhancing characteristics, which in turn was viewed as correlated to amino acid metabolism or enhanced condition at the tissue level. Nonetheless, Waagbø et al. (2010) could not confirm the relationship for adult salmon, and Remø et al. (2014) could not find any such evidence for post-smolt salmon. Condition factor increased from the initiation to termination of the feeding trial, and no differences could be identified between experimental groups or between CTRL and respective sources of His at medium or high levels. Previous studies have varied in their findings related to the His and K factor. Waagbø et al. (2010) and Remø et al. (2014) found no such relationship, while Sambraus et al. (2017) identified that His supplemented in diets reflected increased condition factor and suggested it may inhabit characteristics that are positive for metabolism in salmon. These findings highlighting that nutritional factors most likely did not cause difference in growth for any fish group.

The key organ indexes development for all experimental groups and CTRL were similar to previous nutritional studies (Hevrøy et al., 2015). The KOIs were reduced in all groups given the highest inclusion level of His compared to control and medium levels for the respective His sources, and there was no difference between the His sources. No previous study has suggested a negative relationship between KOI growth and His supplementation to the best of the author's

knowledge. His status has been correlated to superior growth in fish (Breck et al., 2005; Sambraus et al., 2017). Other studies found no evidence of such a relationship between His and growth enhancement at either organ or somatic levels (Remø et al., 2014; Waagbø et al., 2010). Therefore, it is surprising that a slight difference of 0.1-0.3 % His kg d.w. could reduce the examined relative organ indexes in all groups. His and its metabolites differ in concentration and compounds between organs (Remø et al., 2014), and it seems reasonable to assume that the supplementation level affects organs differently. It is known that the amount of His needed to satisfy demands for protein synthesis and growth is well below the amount needed to mitigate cataract (Waagbø et al., 2010) and that high levels of His have the potential to stimulate the production of histidase, which enables His degradation, causing an imbalanced amino acid composition in the diet and increased catabolism (Holecek, 2020). However, a previous study found no evidence of histidase production at high supplementation levels (Remø et al., 2014). The decreased organ indexes in fish fed the highest His levels warrant further research.

Thus, the different His sources investigated in the present study reflected similar feed efficiency and somatic performance, measured as feed intake, FCR, growth, SGR, K factor, and relative organ indexes in Atlantic salmon.

4.2 All histidine sources reduced cataract severity

Low cataract prevalence and severity were recorded at the initiation of the feeding trial. After the 6-week trial period in seawater, both severity and prevalence had increased in all groups. The fish groups fed the non-supplemented CTRL diet, containing 0.93% His/kg developed the highest cataract severity during the feeding trial. Among the experimental diets, fish given the CM and KH diets had lower cataract severity than fish given the KM and AM, while CM was also lower than CH; all others were similar (*Fig 2 and Table 3*). Thus, the cataract severity among His supplementation levels fell with increasing supplementation, irrespective of source. These findings are underscored by regression analysis, which found that the same equation could express all three His sources (*Fig 3*) and identified a relationship where increasing His supplementation decreased cataract severity. Coupled together, these results show that supplementation of all His sources reduce cataract similarly, although fish given the CM diet deviated somewhat.

Increasing cataract prevalence and severity is assumed to be a His deficiency symptom due to its occurrence before growth reduction. Therefore, it acts as a precautious signal of suboptimal dietary His nutrition (Breck et al., 2005; Waagbø et al., 2010). It can also arise due to a dysfunctional osmoregulatory system during smoltification in seawater (Bjerkås et al., 2003). Increased cataract development is often found to materialize in periods such as smoltification, transfer to seawater, and fluctuating temperatures (Bjerkås & Bjørnestad, 1999; Breck et al., 2005; Remø et al., 2014; Sambraus et al., 2017; Waagbø et al., 2010). The minimum requirement for growth of 0.8% His/kg in feed for salmon (NRC, 2011) is not within distance of the amount needed to mitigate cataract in specific periods where the salmon is more susceptible to cataract, such as after transfer to sea or the second summer at sea during a temperature increase (Remø et al., 2014; Waagbø et al., 2010). The high cataract score in CTRL fish reflected this, and signifies that modern diets without supplemented His falls short of fulfilling the demand for His related to biochemical functions beyond protein synthesis, which has also been communicated in previous studies (Breck et al., 2005; Remø et al., 2014; Waagbø et al., 2010).

Variations in growth and, thus, nutritional requirements are often a result of either biological or physical factors and usually confluent with each other (Waagbø & Remø, 2020). The lens concentration of NAH quickly declines when metabolism increases (Sambraus et al., 2017), as it does with increasing temperatures (Hevrøy et al., 2015). The temperature has been proven to

correlate with cataract development in previous nutritional studies, and the underlying driver is assumed to be an acceleration in growth and metabolism dynamics which also causes increased oxidative pressure and shifts in the antioxidant defense system (Remø et al., 2017; Sambraus et al., 2017). The high overall prevalence of cataract in the present study reflects the effect of temperature on cataract development, and the limitations of His supplementation. As such, the optimal temperature used in the present study increased the risk of cataract development during the smoltification and transfer to the sea.

Previous studies have identified a positive correlation between growth and cataract severity (Breck et al., 2005; Taylor et al., 2015; Waagbø et al., 1996). The driver behind this is hypothesized to be the hierarchical prioritization of muscle tissue over lens tissue, creating an antagonistic dichotomy between growth-driven demand for muscle protein synthesis and free anserine and increasing His demand in the lens for protein synthesis and free NAH concentration. However, no correlation between weight and cataract score could be identified in the present study ($P = 0.055$). There seems to be no reason to believe that the His source or His level caused the lower outlier in the cataract score for fish given the CM diet. The results are underscored by the other medium His diets (AM and KM) having a higher cataract score than the respective high His diets (AH and KH) (*Table 3*). Remø et al. (2014) identified the optimal amount of His supplemented in feed to prevent cataracts after seawater transfer, with the optimal His level to minimize cataract severity at 1.34% His/kg and minimized prevalence at 1.44% His/kg. These findings showcase the importance of elevated dietary His levels when the salmon is vulnerable to cataract development. Thus, the different His sources investigated in the present study reflected a similar ability to reduce cataract development after sea transfer of Atlantic salmon.

4.3 Tissue levels correlate with dietary His

Lens free His and NAH concentrations increased for all fish given diets supplemented with His during the six-week feeding trial. The lens free His in fish given the non-supplemented diet was the lowest, while fish given the medium supplemented diets had non-significantly higher levels, but both were significantly lower than fish given the high supplemented diets (*Fig 4*). Lens NAH was lower in fish fed the non-supplemented diet versus both medium and high diets (*Fig* 4), and fish given the high His diets had similar lens NAH as fish given their respective medium supplemented diets. No difference in lens free His or NAH could be found between the three different His sources. The findings were underscored by regression analysis finding a positive relationship between lens free His and NAH and His supplemented to diet (*Fig 5*). In addition, all three His sources could be expressed as the same equation for both free His and NAH. The findings for lens His and NAH reflect the trend seen in feed utilization, fish performance, and cataract development; the three His sources did not differ in their biological efficacy.

Rhodes et al. (2010) identified NAH as an osmolyte in the salmon lens. The osmolyte was found to be the balancing factor in aqueous humor, with osmolality as the variable (Rhodes et al., 2010), and low NAH concentration is defined as a risk factor for cataract development (Remø et al., 2014; Sambraus et al., 2017). In periods of significant fluctuation in osmolality (e.g., transfer to seawater), it is therefore assumed that NAH is especially important. Indeed, in situations where the supply of His is reduced and NAH falls below the critical concentration needed for lens homeostasis, it is assumed that lens proteins aggregate and cataracts develop (Rhodes et al., 2010). Studies performed on the salmon lens in hypo-osmotic conditions using an *ex vivo* model found a correlation between NAH efflux and His supplementation level, in addition to the protein source (Trösße et al., 2009). Due to its superior ability to regulate osmosis in the lens, NAH seems to be preferred by the lens for efflux over other amino acids to prevent tissue disruption (Trösße et al., 2010). Breck et al. (2005) suggested that due to NAH's nature in balancing water volumes, it seemed reasonable to assume that the aqueous humor osmolality was correlated to plasma level ions and affected by external changes in osmolality such as seawater transfer. In the present study, the mean NAH levels for medium diets was 8.0 µmol NAH/g, and for the high diets was 10.0 µmol NAH/g. In a similar study, Remø et al. (2014) found NAH levels of 2 – 13 µmol/g, on par with the present study, while Sambraus et al. (2017) found somewhat lower levels of 5.0 – 7.0 µmol/g. Remø et al. (2014) estimated that the level of NAH in the lens needed to reduce prevalence and severity of cataracts was 10.8 µmol NAH/g

lens and 8.8 µmol NAH/g, respectively. Thus, the levels of NAH were slightly below the level assumed to be optimal for reducing prevalence in high diets but well above the level needed to reduce the severity. At the same time, the medium diets had levels slightly below the optimal level needed to reduce the severity and well below the level needed to reduce prevalence. The low level of both lens free His and NAH found in fish fed the CTRL diet, which was almost unchanged from the initiation to termination of the nutritional trial, shows the importance of His supplementation to support lens free His and NAH in the period after seawater transfer. The findings suggest that reducing the His supplementation in the feed below 1.4% His/kg during seawater transfer can cause increased cataract prevalence, severity, and reduced fish welfare, irrespective of His source. Thus, the different His sources investigated in the present study reflected the same concentrations in lens tissues and the same biological efficacy, following the patterns seen in previous studies on His supplementation (Remo et al., 2014; Sambraus et al., 2017).

Heart free His and NAH increased for fish fed His supplemented diets from the initiation to termination of the feeding trial. The concentration of heart free His and NAH reflected the same pattern as was seen for lens tissue, with a positive relationship between the level of His supplemented and concentration of free His and NAH. Differences in heart tissue concentrations were found between fish given the non-supplemented diet and supplemented diets and between medium and high supplemented diets. No difference was found between His sources. The heart His and NAH levels in supplemented diets ranged from 0.5 - 1.0 µmol/g and 2.5 - 3.0 µmol/g, respectively. In a similar study, Remø et al. (2014) found heart His and NAH levels of 0.2 – 1.2 µmol/g and 1.9 – 3.5 µmol/g. In the present study, both heart His and NAH seemed to plateau around 1.2% His/kg.

These findings are consistent with earlier studies on dietary His and Atlantic salmon. Remø et al. (2014) found the same increase in the heart free His and NAH after seawater transfer and a correlation between heart free His and NAH levels and the amount of supplemented His. However, during the following weeks, a plateau was identified for NAH, and it was assumed that heart NAH reached a saturation point somewhere between 5 and 13 weeks after seawater transfer (Remø et al., 2014). In this study, fish given the KH diet achieved the highest heart free His and NAH, which also had the highest level of His supplemented. However, since increasing the level of His supplemented had little effect on heart free His and NAH in previous studies

(Remø et al., 2014), this may question the effect of increasing His supplementation to elevate heart concentrations and the usefulness of heart His and NAH as His status markers. The different His sources investigated in the present study reflected the same concentrations in heart tissues and the same biological efficacy in Atlantic salmon.

Both muscle free His, carnosine, and anserine increased in fish given His supplemented diets from the initiation to termination of the feed trial. Fish given the unsupplemented diet saw a depletion of anserine in muscle tissue, while free His was stable. Both free His, carnosine, and anserine correlated positively with the level of His supplementation in feed, and no difference was found between His sources. β-alanine correlated negatively with the level of His supplemented and decreased from the initiation to termination of the feeding trial, and no difference was found between His sources. 1-methyl-His concentrations in white muscle tissue, were stable throughout the study and similar for all diet groups. These findings support that even the highest supplementation levels did not induce degradation of a possible excess of His in the muscle, in line with a previous study that found no sign of liver histidase at elevated His nutrition (Remø et al., 2014).

In the present study, the anserine levels ranged from 17 – 19 µmol/g. Remø et al. (2014) found slightly lower anserine levels, ranging from 9 – 14 µmol/g, while Sambraus et al. (2017) found similar levels. The levels of carnosine and β-alanine were somewhat higher in this study compared to Sambraus et al. (2017), which found that fish provided with high His diets had Carnosine and β-alanine levels of 0.32 – 0.59 µmol/g and 0.30 – 0.45 µmol/g, respectively, compared to 0.30 – 1.04 µmol/g and 0.34 – 0.80 µmol/g for this study. However, Remø et al. (2014) found carnosine and B-alanine levels in line with those found in the present study. The Atlantic salmon given the three His sources achieved similar and satisfactory tissue levels of His and its metabolites.

Both anserine and NAH appear in an order of magnitude higher than other His compounds and amino acids in the muscle and lens, respectively (*Figs 4, 8, and 10*). Anserine is the most important imidazole compound in the Atlantic salmon white muscle, both in concentration and physiological function (Abe, 1987; Ogata et al., 1998). Furthermore, the concentration of the muscle tissue has been found to correlate with His supplementation in the diet (Remø et al., 2014). The depletion of both free His and anserine in muscle tissue of fish given the

unsupplemented diet reflects how the increase in body size with growth necessitates the His supply to continuing recruitment of muscle fibers in juvenile fish (Johnston et al., 2006). In studies feeding Atlantic salmon with His supplemented diets, Tröße et al. (2010) found anserine levels in muscle tissue were not as correlated to the supplemented His levels compared with lens NAH. It was concluded that this could point towards a picture where His is more available in the blood circulation for white muscle tissue than the lens, or due to cumbersome imidazole supply the lens, as discussed earlier (Tröße, 2010). Furthermore, it was found that a negative correlation existed between cataract score and muscle anserine concentration, highlighting that dietary His supplemented required to minimize cataract risk coincides with saturation in levels needed for anserine synthesis (Remø et al., 2014). Previous nutritional studies concentrated on His have identified evidence supporting a hierarchical distribution of free His within the salmon, where protein synthesis and anserine seem to be prioritized over NAH (Remø et al., 2014; Sambraus et al., 2017). Such a prioritization makes sense from an evolutionary standpoint due to anserine's buffering capabilities during burst-swimming, which often is essential for escaping predators or catching prey (Ogata et al., 1998). Thus, it seems that salmon has a priority hierarchy for His that prioritizes delegation of free His to protein synthesis, muscle anserine, and finally NAH to the lens. It may also reflect the cumbersome way for free His to reach the lens through multiple eye tissue borders compared to the comparatively larger muscle tissue. Remø et al. (2014) found that maximum muscle anserine concentration was reached at the same level as where supplemented His started to mitigate cataract severity – highlighting the possibility of such a hierarchy within the salmon metabolism (Remø et al., 2014).

The level of β-alanine correlated negatively with increasing levels of His supplemented in the present study. Previous studies found similar results, and it is reasonable to assume that the elevated levels of β-alanine are related to suboptimal His nutrition in salmon (Remø et al., 2014). The assumed link between β-alanine and suboptimal His nutrition is due to β-alanine being a a precursor in the synthesis of His to carnosine and further methylation to anserine, and therefore seems to increase when His is not available for anserine synthesis. Thus, the different His sources investigated in the present study reflected the same concentrations of free His and imidazoles in muscle tissues and the same biological efficacy in Atlantic salmon.

5. Conclusion

Given the three His sources supplemented in up-to-date industry feed, Atlantic salmon post-smolt had similar FCR and achieved similar feed intake, Somatic weight, SGR, K-factor, and somatic indexes. The supplementation level of His did affect somatic indexes negatively.

The ability of the three different His sources to reduce cataracts was compared, and no dissimilarities were found between His sources. His supplementation correlated negatively with cataract score. The necessity for high His supplementation levels for optimal cataract mitigation was confirmed.

The His supplementation correlated positively with His and NAH in the lens and heart. The muscle levels of His, anserine correlated positively with His supplementation, while β-alanine correlated negatively. No difference was found for 1-methyl-histidine.

The lens and heart tissue levels of His and NAH, and the muscle tissue levels of His, anserine carnosine, β-alanine, and 1-methyl-histidine were similar in fish given all three His sources.

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