Gastrointestinal Physiology of Chinook Salmon, *Oncorhynchus tshawytscha* (Walbaum) with Gastric Dilation Air Sacculitis (GDAS)

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by

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General Abstract

The syndrome known as Gastric Dilation Air Sacculitis (GDAS) has recently been described by Lumsden *et al.* (2002) for Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum), in seawater (SW) culture in New Zealand. The syndrome is characterised by distended abdomens, gastric dilation and air sacculitis, increased feed conversion ratios (FCR) and mortality. Consequently, financial returns on affected stocks are greatly reduced. A study into the epidemiology and physiology of the syndrome was initiated, working with the major aquaculture company, The New Zealand King Salmon Company (NZKS). The study revealed causative factors of GDAS. GDAS was experimentally induced only in saltwater by feeding a commercially manufactured low-cohesion pelleted diet. Control groups were fed a different diet with high physical cohesion. Low-cohesion pellets have previously been associated with a high incidence of GDAS in commercial sea cages. These data implicated osmoregulatory stress and physical properties of the feed in GDAS development. In addition, gastrointestinal (GI) physiology in GDAS -affected and -control fish was characterised. The process of GDAS development in *O. tshawytscha* is characterised by a loss of smooth muscle tone of the stomach as it distends. Laplace's law (P= 2T/r, where P is the distending pressure, T is the tension in the wall and r is the radius of the cylinder) predicts that unless muscle mass increases, the ability of the stomach wall to contract will be lost and consequently a loss of GI motor function will result. Therefore, GI circular smooth muscle integrity in terms of (1) stimulated and maximal contractility, (2) osmoregulatory ability of the intestine and the (3) control of the GI system was studied in pathologically affected (+ve) and unaffected (-ve) smolt. Affected fish showed changes in GI circular smooth muscle function and

osmoregulatory dysfunction. Feeding different diets induced distinct gastric evacuation patterns. The intestinal brake hypothesis is presented and argued to be the probable mechanism for GDAS development. GDAS (+ve) serum showed the presence of factors capable of contracting gut smooth muscle. In addition, potential humoral mediators of the intestinal brake in fish were investigated.

CHAPTER 1

1.1 Background on GDAS and NZ Salmon Aquaculture

The syndrome known as Gastric Dilation Air Sacculitis (GDAS) has recently been described by Lumsden *et al*. (2002) for Chinook salmon (*Oncorhynchus tshawytscha,* Walbaum), in SW culture in New Zealand. The syndrome is characterised by distended abdomens and gastric dilation and air sacculitis, increased feed conversion rates and increased mortality (Lumsden *et al*. 2002). Consequently, aquaculture operations growing Chinook affected by GDAS in New Zealand waters have greatly reduced financial return on those fish. Since GDAS has recently been quite prevalent in some SW operations in New Zealand, research into the syndrome was a big industry priority. Gastric dilation also occurs in Canadian and European salmonid farming operations from time to time and is commonly referred to as 'bloat'. The condition described by Lumsden *et al*. (2002) is similar to diseases described for rainbow trout, (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) in Norway and from Chinook salmon in British Columbia, Canada (Lumsden *et al*. 2002). The syndrome has also been reported to have been induced experimentally in rainbow trout fed diets with high doses of histamine incorporated over a 6-10 week period (Wantanabe *et al.* 1987). Evidence for temperature stress-induced gastric dilation (0.5°C chilling for 12 hours in starved fish) was also presented by Rórvic *et al.* (2000), and it appears that high temperatures may exacerbate the syndrome (Lumsden *et al*. 2003; NZKS pers. com.) probably due to the Q_{10} effect.

Post-mortem dissection of GDAS affected fish in the Lumsden *et al*. (2002) study revealed a dilated and flaccid stomach without visible rugal folds, typically containing copious oil, fluid and feed slurry (chyme). Air sacculitis was described as a thickened,

dilated swimbladder with a mixed mucosal inflammatory infiltrate and a luminal exudate associated with large numbers of morphologically diverse bacteria (Lumsden *et al*. 2002). The swimbladder content varied in viscosity and volume. Furthermore, gastric dilation or air sacculitis was reported to occur alone or together in the same fish.

1.2 General physiology of Chinook Salmon

1.2.1. Life History

Chinook salmon is one of the most widely distributed species of salmon in the eastern North Pacific Ocean. They range from the Sacramento River in California to the Kamchatka Peninsula in Russia. Chinook have been introduced to lakes and rivers far outside of their home range, including New Zealand (Healey, 1991).

Salmon life history is complex, being split into several distinct stages (Figure 1.1). Chinook eggs hatch in freshwater (FW) as alevin and progressively develop into fry, parr and then smolt. Smolt display different physiology to earlier stages and is typically the stage where the fish move from FW into SW (Healey, 1991). Smolt, fry and parr are known to inhabit brackish waters in their natural range (Healey, 1991).

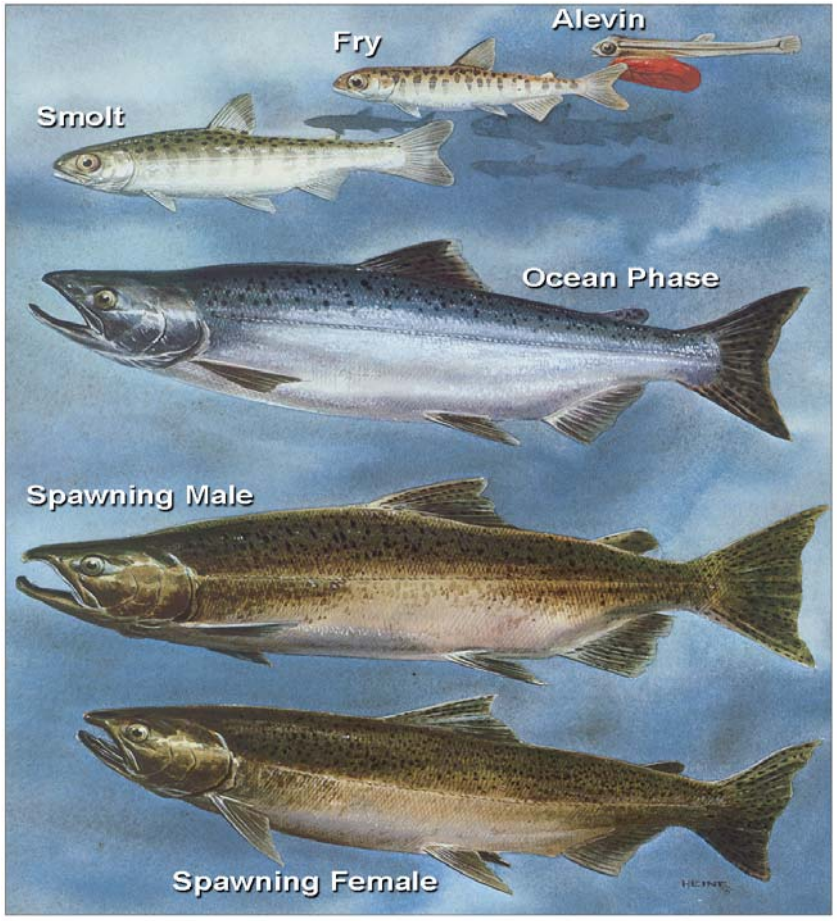

Figure 1.1 Chinook Life History stages (adapted from Healey, 1991).

Chinook are anadromous, ubiquitously hatching in FW and developing for a variable length of time before moving into SW. These are known as 'ocean-type' Chinook. Smoltification involves the up regulation of Na^+/K^+-ATPase in the chloride cells of the gills and the epithelium of the intestine (McCormick, 2001). An energy requiring enzyme, Na^+/K^+-ATPase creates electrochemical gradients across cell membranes, which drive the active transport of plasma ions. Further information is provided in Chapter 2.

Homeostasis & Smoltification

During the parr-smolt transformation whole-animal water tolerance to seawater is increased (Usher, 1988). These changes are associated with changes at the cellular (epithelial Na^+/K^+-ATPase expression) (McCormick, 2001) and whole animal level (metabolism, behaviour and growth) (Usher, 1988). In SW, the osmotic pressure of blood is about one third that of the external medium, so salmon must take up water and excrete excess salts (Withers, 1992). Smolt develop the ability to take up osmotically free water in order to maintain water and ion balance in SW. Water is obtained by ingesting SW, and then actively taking up the dissolved ions into the blood. Water is then drawn across the intestinal epithelium by a number of pathways (Usher, 1988). Excess sodium (Na^+) and chloride ($Cl.^-$) ions are excreted at the gill (Usher, 1988).

As in other marine teleosts, the intestine is the major site of water uptake in salmonids living in the sea. Shehadeh and Gordon (1969) were the first to show increased drinking rates in rainbow trout following transfer to SW from FW. Pyloric caecae are unique out-pocketings of the fish intestine. Found in many species, caecae are located immediately distal to the pyloric sphincter of the stomach (Viellette, 2004). Their number varies greatly with species and as many as 200 caecae are present in salmonids, accounting for roughly 70% of the nominal surface area of the post-gastric intestinal tract (Viellette, 2004).

Up until very recently, the pyloric caecae of the fish intestine were not recognised as important sites of osmoregulation. In 2004, it was shown for the first time that pyloric caecae are very important osmoregulatory organs (Viellette, 2004). Viellette (2004) made direct volumetric measures of the fluid uptake rate which he showed to be increased in

SW-adapted smolt relative to FW-adapted smolt. He also correlated increased Na^+K^+-ATPase activity with the SW smolt.

Chinook can also remain in FW past the smolt life-history stage. These are known as 'stream-type'. Their osmoregulatory physiology reverts back to parr-like physiology (Healey, 1991; Viellette, 2004). The New Zealand stocks display both 'stream-type' and 'ocean-type' life-histories (Taylor, 1991), similar to their parent stocks from the Sacramento River (Quinn, 1996). The aquaculture industry in New Zealand reflects the variable nature of Chinook physiology with both FW and SW operations. Some, such as those of NZKS operate FW hatcheries, later transferring the smolt into SW for on-growing in the ocean. Most other companies raise adult fish in FW.

Commercial sea-cage culture by NZKS begins with harvesting eggs and sperm from FW brood-stock. These eggs are fertilised and on-grown in FW until they reach the smolt life-history stage. After freighting in brackish water, smolt are transferred straight into SW (NZKS; pers. com.), where they must acclimate to their new hypersaline environment. Fish are then on-grown in high density cages on maximal feeding regimens (automatic, camera operated). Since salmon will feed to satiation (Lovell, 2002), their stomachs are almost always full with feed pellets.

1.2.2. Diet

Chinook are voracious visual feeding carnivores (Lovell, 2002). In their natural environment, just as in their adopted homes in New Zealand, Chinook fry and parr eat a mixture of terrestrial, FW and estuarine invertebrates and fish (Quinn, 1996). Higgs et al. (1995) summarise the data from 2756 fry stomachs from 12 studies of wild caught fish. Typical stomach contents included amphipods, insects, copepods, mysids and euphasiids and cladocerans. Higgs *et al*. (1995) also summarise wild caught smolt gut contents (13 studies; n=1258). Typical smolt stomach content included salmon fry, amphipods, decapod larvae, copepods and multiple insect orders. Galaxiid fish have been found in the stomachs of fish sampled from both Australia and New Zealand (Quinn, 1996). Smolt diet was shown to reflect their environment in FW, estuarine and SW environments. Adult fish feeding (17 studies; n=5687) is also reviewed by Higgs *et al*. (1995). The stomach contents were comprised primarily of fish, euphasiids, crab megalopa and zoea, squid and amphipods. All are large solid food items ('high cohesion'), very different from the manufactured fine particulate, high nutrient pellets commonly fed in commercial salmon operations worldwide.

Salmon aquaculture operations feed pelleted feed manufactured by a number of methods; the two most common being extrusion and steam pelleting (Halver, 1989). These are typically referred to as 'dry feeds'. Both of these processes utilise extremely fine ground particles from multiple ingredient sources. These ingredients are sourced as available and the final products often differ in terms of ingredient source between batches (NZKS pers. com; Halver, 1989). However, feed composition, as determined by proximal analysis, is remarkably consistent between manufacturers and usually conforms to the

specifications of the client (NZKS; pers.com). Typical feed contains about 40% protein, 25% lipid, 25% carbohydrate and 10% water, with the remainder being made up by vitamins and minerals and other additives (Hardy *et al*. 2002). When exposed to moisture the pellets disaggregate at varying rates dependent on moisture content and degree of starch gelatinization (Jobling, 1987).

1.3 Gastrointestinal Physiology of Chinook Salmon

The GI system of the fish operates in a similar way to that of other vertebrate groups (Olsson and Holmgren, 2001). Organ systems vary, but primary function and biochemistry of the alimentary canal are remarkably conserved in higher vertebrates (Withers, 1992). The Chinook salmon GI tract is typical of most teleost species, and its genus (*Oncorhynchus*) is well represented in the literature. Hence, *Oncorhynchus* digestion is relatively well understood in relation to other teleost species. However, there are still large gaps in the understanding of the teleostean gastrointestinal physiology.

As mentioned, Chinook are voracious visual feeding carnivores (Lovell, 2002). They will feed till satiation if fed *ad lib* (Higgs, 1995). In saltwater they must also drink continuously (Shehadeh and Gordon, 1969). Ingested food items and water are passed from the mouth, through the oesophagus to the cardiac stomach by swallowing, where the food is chemically (HCl and digestive enzymes from the gastric epithelia) and physically (via smooth muscle contractions) degraded in the stomach (Barrenechea *et al*. 1994). In so doing, digesta particle size is reduced (Barrenechea *et al*. 1994). Upon mechanical and chemical degradation, the cardiac stomach moves fine particle digesta to the pyloric stomach via peristalsis for further degradation (Olsson and Holmgren, 2001). From there,

digesta and ingested water are periodically passed through the pyloric sphincter, by propagating peristaltic waves reaching the pylorus (Davenport, 1989), into the proximal intestine (pyloric caecae and mid-gut) for nutritive, ion and H_2O uptake (Weisbrodt, 1991; Viellette, 2004). Dehydrated faeces and a small volume of urine (containing Mg^{++} and NH_4) are expelled through the rectum and out the anus (Sveier, 1999). The pyloric sphincter is known to contract at the end of the peristaltic wave (Ganong, 1977). At low levels of exogenous stimuli, chyme will be forced through. However, in the presence of powerful stimuli, the sphincter will contract with sufficient force to stop the chyme passing through. In addition, air is ingested by salmon in both FW and SW environments to control buoyancy. The air is passed from the oesophagus into the swimbladder via the pneumatic duct. This system is regulated by pneumatic duct smooth muscle tone (Withers, 1992).

The GI system of vertebrates, with fish being no exception, is extremely complex in terms of its control mechanisms (Olsson and Holmgren, 2001). A whole host of humoral factors and neural stimuli control motility patterns, digestion and uptake. Many of these humoral factors have multiple effects throughout the body (Ruppin and Domschke, 1980). Refer to Chapter 3 for a more in depth discussion of the control of the fish gastrointestinal system.

1.4 Rationale and Aims of the Study

Lumsden *et al*. (2003) suggested that both the commercial feed pellet and osmoregulatory stress post-transfer were potentially causative agents of GDAS. The current study concentrates on these ideas and focuses on feed cohesion properties, particle size and SW osmoregulation. There was no conclusive link shown between GI smooth muscle atrophy and osmoregulatory dysfunction and GDAS development in earlier work (NZKS; unpublished data). Rather, NZKS characterised changes in cellular morphology and correlated this with gross pathology. Feed properties (cohesion) were anecdotally correlated with increased GDAS prevalence in subsequent NZKS trials. From this work came the first suggestion that an 'ileal brake type' dysfunction may be occurring in GDAS +ve fish (Ben Wybourne, pers. com.). The ileal brake is a feedback mechanism known to occur in mammals, resulting in the slowing of gastric evacuation from stomach to intestine, mediated by the pyloric sphincter (Dobson *et al.* 1998; Vu *et al*. 2000). The sphincter is under humoral and nervous control (Heading, 1980). The ileal brake is activated locally by the presence of nutritive chyme (e.g. amino and fatty acids (Dobson *et al.* 1998)). Some nutritive molecules are more potent stimulators of the ileal brake, for example, fatty acids increase in their potency of inhibition of gastric emptying with molecular chain lengths above 8 to a maximum of 14 in mammals (Heading, 1980). Amino acids seem to be equal in their ability to inhibit gastric emptying, except for tryptophan which induces a marked slowing of gastric emptying in the dog (Heading, 1980). Investigations of the ileal brake have been conducted in humans, rats, guinea pigs, rabbits, dogs, and pigs (Dobson *et al.* 1998).

An ileal brake type system, termed the intestinal brake, operates in teleost fish and has been shown experimentally to slow gastric emptying in a similar manner to that in mammals (Olsson and Holmgren, 2001). Dysfunction of this mechanism is hypothesised in the current study to result in a marked slowing of gastric emptying, due to an overactive feedback loop acting on the pyloric sphincter. The cause of this is suggested to be a result of commercial feed pellet characteristics that alter the rate of gastric emptying of liquid and solid digesta in osmoregulatory stressed (SW acclimated and acclimating) *O. tshawytscha*) to produce GDAS.

This thesis aims to close the gaps in the understanding of the syndrome by (1) inducing GDAS (Chapter 2), (2) characterising changes in gastrointestinal and osmoregulatory physiology of GDAS +ve fish relative to control fish (Chapter 2) and by (3) investigating possible mechanisms of its development (the intestinal brake hypothesis) (Chapters 2 and 3).

Working with NZKS information and the Wybourne proposal, gastrointestinal feedback-loop dysfunction is formally defined here as the 'intestinal brake hypothesis'. This hypothesis was adopted as a potential mechanism of the syndrome and accordingly tested for by the application of GDAS +ve serum to smooth muscle strips *in vitro* (Chapter 3). Potential humoral factors hypothesised to be involved as intermediate signalling molecules in the intestinal brake were also investigated (Chapter 3). In so doing, the first report of the effects of these peptides on Chinook GI tissues are presented.

1.5 The Intestinal Brake Hypothesis

The intestinal brake is an adaptation of the term ileal brake (mammals) as it applies to the fish gut, which lacks an ileum. Simply explained, the intestinal brake hypothesis proposes that a malfunctioning ileal-brake type mechanism exists in Chinook fed highly unstable (low-cohesion), fine particulate feed pellets in SW. As outlined, the ileal brake in mammals is activated when high fat and protein meals are ingested. Post-prandially slowed gastric evacuation (slowed peristalsis and increased pyloric sphincter tonus) results, which is mediated by GI peptides and the CNS (Liddle, 1986).

Since teleosts in SW must constantly obtain osmotically free water, they must therefore continuously drink the external medium in order to actively uptake water *via* ion-linked H_2O transport across the intestinal epithelia (Withers, 1992). This compensates for the continual loss of H_2O across the gills in a hypersaline medium.

It is proposed that in GDAS affected fish, the movement of water and chyme into the pyloric caecae of the intestine is obstructed by prolonged increase in the tonus of the pyloric sphincter, that is above normal intestinal brake levels, as it contracts at the end of propagating peristaltic waves in the stomach (discussed in Chapter 3). This is thought to restrict chyme and water movement between the stomach and intestine below the typical level associated with the intestinal brake in healthy fish. Therefore, it is suggested that only once nutritive load on the intestine is reduced can increased motility patterns begin. It is likely that this will rarely occur in SW aquaculture operations feeding maximally. Since the thirst response is always active in SW teleosts, large volumes of SW are ingested (Shehadeh and Gordon, 1969). Obstructed movement of ingested water is thought to increase intramural pressure in the stomach, as the gastroesophangeal

sphincter and the pyloric sphincter will prevent stomach contents from escaping. Since *O. tshawytscha* is a physostomous species, reflux of stomach contents into the swimbladder potentially occurs. This is thought to result in the air sacculitis observed in chronically affected GDAS +ve fish. The intestinal brake hypothesis is shown schematically overleaf.

In summary, maximal feeding combined with a low-cohesion pelleted diet and osmoregulatory stress are hypothesised to result in GDAS manifestation in commercial cage salmon aquaculture via the 'intestinal brake hypothesis'.

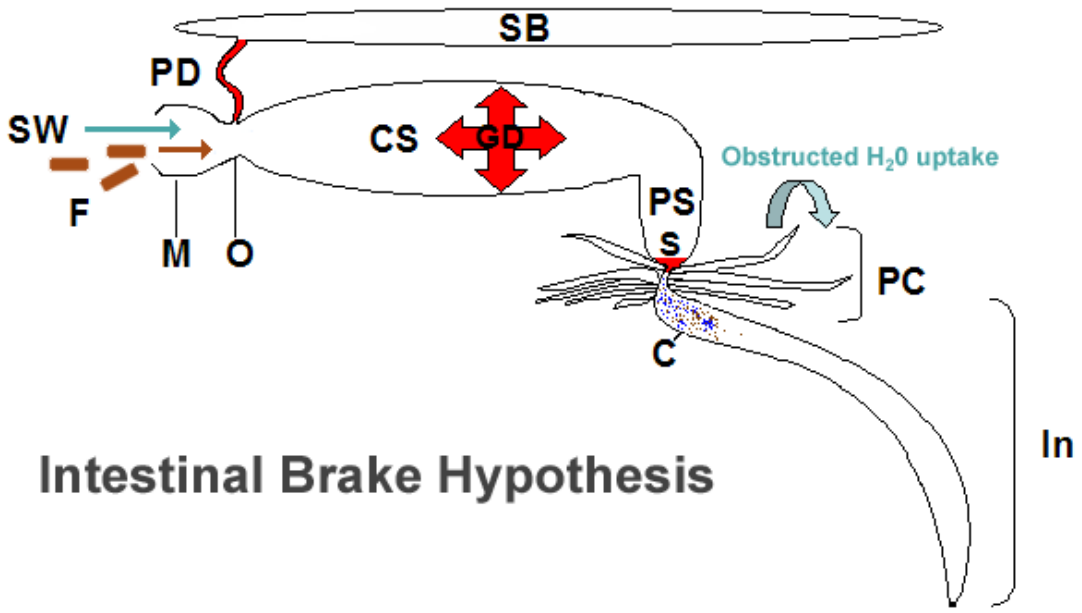

Figure 2. The IBH: Low-cohesion, fine particulate feed **(F)** and saltwater **(SW)** are taken into the mouth **(M)**, passed through the oesophagus **(O)** into the cardiac stomach **(CS)**. Rapidly, a high-nutrient chyme **(C)** forms, passing through the pyloric stomach and pyloric sphincter via peristalsis. The chyme then arrives in the pyloric caecae **(PC)**/intestine **(In)** activating sustained PS tonus at the end of propagating peristaltic waves, via nutritive chemoreception. Gastric dilation **(GD)** results due to the elevated pyloric sphincter **(PS)** tonus and large volume of water ingested Chyme and bacteria from the stomach are refluxed into the swimbladder **(SB)** via the pneumatic duct **(PD)** due to high intraluminal pressure in the stomach, resulting in Air Sacculitis.

1.6 Statement of Hypotheses

1. GDAS is able to be induced only in SW with a low cohesion diet.

2. GDAS impairs osmoregulatory ability in SW.

3. GDAS does not simply result in a loss of smooth muscle control:

   - Cardiac stomach contractility decreases with GDAS onset (Laplace's law).
   - Pyloric sphincter contractility increases with GDAS onset.

4. Experimentally induced GDAS histology correlates with commercial cage GDAS histology.

5. Rates of gastric evacuation in healthy fish stomachs differ when feeds of low and high cohesiveness are fed.

6. GDAS +ve serum carries agents capable of causing GI dysfunction.

7. Candidate humoral factors produce patterns of slowed motility in isolated GI tissues.

CHAPTER 2

Gastrointestinal Smooth Muscle Integrity and Osmoregulation in Seawater Adapted Chinook Salmon (*Oncorhynchus tshawytscha*) Smolt with Gastric Dilation Air Sacculitis: Epidemiological Correlates with Feed, Gastric Evacuation and Osmotic Environment

2.1 Abstract

NZKS feed trials anecdotally report low cohesion diets correlate with GDAS incidence in SW trials (unpublished data). Therefore, GDAS epidemiology and physiology, in terms of GI smooth muscle integrity and osmoregulation were investigated. This was done by feeding two experimental diets in two separate trials. Diet A (low cohesion pellets) and Diet B (high cohesion pellets) were fed to both FW- (n=1000) and SW-adapted fish, (n=640) with different acclimation histories. GDAS was induced in the SW trial by feeding diet A but not diet B. The primary symptom of GDAS is gastric dilation (Lumsden *et al*. 2002). Therefore, this aspect of the syndrome was investigated. As the process of GDAS-associated distension is hypothesised to result in the loss of smooth muscle tone of the stomach and an increase in the integrity of pyloric sphincter, maximal force development of cardiac stomach and pyloric sphincter circular smooth muscle was investigated. This was measured in response to acetylcholine (ACh) and potassium chloride (KCl), in GDAS affected (+ve) and unaffected (–ve) fish in both FW and SW adapted Chinook smolt. Wet tissue mass standardised (TMS) cardiac stomach smooth muscle contractility in response to $1x10^{-4}$ M ACh in fish fed diet A was significantly reduced relative to controls in weeks 3 (P=0.0217), 4 (P=0.0425) and 5 (P=0.0310). TMS cardiac stomach (CS) smooth muscle contractility in response to $1x10^{-3}$ M ACh in fish fed diet A was also significantly reduced relative to controls in weeks 3 (P=0.0069) and 5

(P=0.0305). TMS CS smooth muscle maximal contractility in response to KCl in fish fed diet A was significantly reduced relative to controls in weeks 3 (P=0.0435), 4 (P=0.0164) and 5 (P=0.0270). Conversely, TMS maximal pyloric sphincter (PS) circular smooth muscle contractility in response to KCl was significantly elevated in fish fed diet A relative to diet B in weeks 4 (P=0.0451) and 5 (P=0.0026), which accords with the intestinal brake hypothesis. Osmoregulatory ability of GDAS +ve and -ve fish was characterised by two methods; (1) measurements of serum osmolality and (2) fluid transport from mucosal to serosal surface of isolated pyloric caecae *in vitro*. The ability to maintain osmoregulatory homeostasis in GDAS +ve fish was impaired. This was reflected in the data: serum osmolality in GDAS developing fish was elevated in smolt in SW after 2 (P=0.0019), 3 (P=0.0012), 4 (P<0.0001) and 5 (P=0.0024) weeks, when compared to controls using unpaired two-tailed Student's t-tests. A reduced fluid transport rate in GDAS +ve fish pyloric caecae was also shown in fish fed diet A in week 3 (P<0.0001), week 4 (P<0.0001) and week 5 (P<0.0001) in SW. In addition, gastric evacuation from the stomach of healthy fish was shown to be significantly different when diets of low (A) or high (B) cohesion were fed. The amount of digesta present in the stomachs of fish fed diet A was found to be significantly lower, relative to controls, 4 hours after feeding (P=0.0075) and significantly higher after 24 hours (P=0.0077). Furthermore, the pattern of evacuation was also shown to be different in fish fed the two diets. Fish fed diet B in SW showed a linear trend of gastric evacuation, while fish fed diet A in SW showed a reverse exponential pattern of evacuation. This work suggests that (1) GDAS may be caused by low pellet cohesion and osmoregulatory stress in SW

(copious drinking of the medium), and (2) GDAS +ve fish show changes in smooth muscle contractility and osmoregulatory homeostatic ability.

2.2 Introduction

Lumsden *et al*. (2003) have suggested that both the nature of the commercial feed pellet fed and osmoregulatory stress post-transfer may be causative agents of the syndrome. They refer to an earlier suggestion that biogenic amines, inadvertently included in feed pellets, may cause GDAS. However, based on their results, Lumsden *et al*. (2003) were able to refute this hypothesis. Later, NZKS commissioned internal investigations into GDAS incidence and its association with feed properties (unpublished data). Feeding trials were conducted with a diet associated with high GDAS incidence in commercial sea-cage operations (NZKS; pers. com.). Their work suggested that the cohesiveness of feed pellets in the stomach is correlated with GDAS incidence. The current study concentrates on these ideas and focuses on feed cohesion properties and osmoregulatory stress being causative agents in GDAS. One of the aims of this project was to induce GDAS by feeding a low-cohesion diet in SW. In order to test the role of feed and osmotic environment in GDAS development, FW and SW feeding trials were conducted. Once fish were deemed to be GDAS +ve, investigations of GDAS +ve physiology were conducted. The physiological parameters measured were (1) GI smooth muscle integrity, (2) intestinal osmoregulation, (3) gastric evacuation and (4) histology of the gut wall. These data were compared with GDAS -ve fish in both SW and FW.

2.2.2 Smooth Muscle Integrity

The process of GDAS development in *O. tshawytscha* is characterised by a loss of muscle tone of the stomach as it distends. Extrapolating from Laplace's law, the increased radius of a cylinder (the stomach) will require an increase in muscle mass to achieve the same degree of contraction. If the muscle mass does not increase then muscle tone will be lost. Muscle integrity was hypothesised to be associated with GDAS onset and a vital component of the intestinal brake hypothesis (Chapter 1), therefore smooth muscle contractility in cardiac stomach was investigated in GDAS +ve and GDAS -ve fish. Furthermore, the intestinal brake hypothesis predicts that pyloric sphincter contractility might increase through powerful and prolonged contractions in GDAS developing fish. An *in vitro* gut ring myography apparatus was utilised in order to test circular smooth muscle contractility, in response to pharmacological application of acetylcholine (ACh) and potassium chloride (KCl). In the mammalian gastrointestinal tract, it has long been known that ACh and KCl produce smooth muscle contraction by their depolarising action (Sanders and Ozaki, 1994).

2.2.3 Osmoregulation

As mentioned, osmoregulation is an important aspect of homeostasis (Withers, 1992). Plasma osmolality must be maintained for proper cellular and whole animal function. Teleost blood is typically in the range of 290-340 mOsmol/L (Oide and Utide, 1968). FW is typically 0-50 $mOsmol.L^{-1}$, while the SW value is typically around 1000 $mOsmol.L^{-1}$ (Smith, 1995). Consequently, in teleost fish living in FW, H_2O is gained and salts are lost via osmosis (Withers, 1992, Smith, 1995). In SW, H_2O is lost and salts are gained.

(Withers, 1992; Smith, 1995). All SW teleosts must regulate their plasma osmolality, but have varying degrees of salinity tolerance (Oide and Utide, 1968). anadromous fish, such as the Chinook salmon, move between FW and SW. This may occur multiple times in the life of the animal and is related to life-history. These animals have the ability to regulate the osmotic pressure of their plasma in variable salinity environments (Healey, 1991).

FW Osmoregulation

Salmonid osmoregulation in FW is shown schematically in Figure 2.1.

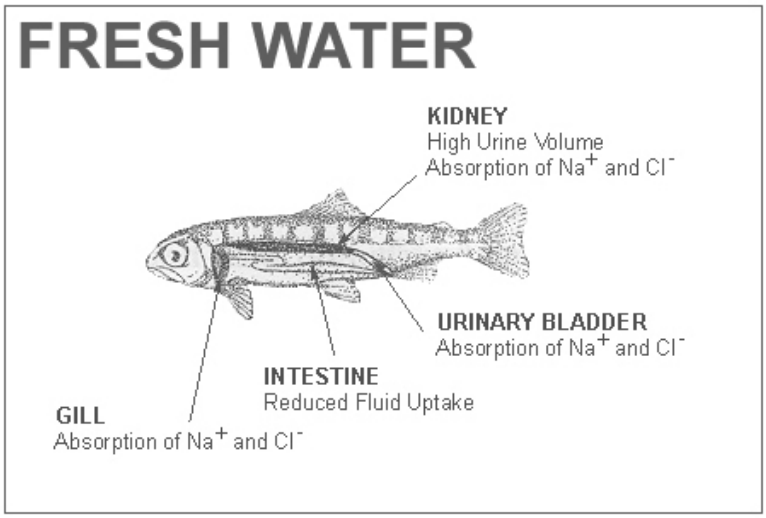

Figure 2.1. Osmoregulation in a FW salmonid (stream-type Chinook) (modified from Healey *et al.* (1991)).

Processes occurring in hyperosmotic FW teleosts include the diffusional loss of salts and the diffusional influx of H_2O across the gills (Clarke and Hirano, 1995). Homeostasis is maintained by the active uptake of Na^+ and Cl^- (monovalent ions) at the gill surface and the production of copious urine (hyposmotic to plasma) (Rawdon and Cornish, 1973; Withers, 1992; Clarke and Hirano, 1995).

Teleosts either do not drink the medium in FW or show reduced rates of drinking relative to SW (Shehadeh and Gordon, 1969; Perrott *et al.* 1992). The intestinal brake hypothesis (Chapter 1) predicts that a high level of fluid intake is required for GDAS development. I wished to test whether FW conditions (minimal drinking) and low cohesion feeds would result in GDAS manifestation. I hypothesised that GDAS would not develop in FW, regardless of feed properties. The FW trial planned in the current study tested these hypotheses by feeding diets of low and high cohesion.

SW Osmoregulation

As described in Chapter 1, Chinook smoltification is accompanied by a marked increase in osmoregulatory ability in SW (Viellette, 2004). Teleosts in SW are hyposmotic to the medium and consequently lose H_2O and gain salts across the gills (Healey, 1991). Teleosts in SW typically lose 11% of total body weight per day ($BW.d^{-1}$) (7.9% osmotic, 0.4 urinary, 2.7 faecal) in H_2O from their blood via osmosis (Withers, 1992). The anadramous Chinook has the ability to regulate the osmotic pressure of its plasma (Higgs *et al.* 1991). This is achieved in SW by utilizing transepithelial enzymes and ion-channels in the gills and intestine (Shehadeh and Gordon, 1969; Rawdon and Cornish, 1973; Withers, 1992). These processes are energetically expensive and usually ATP dependent (Withers, 1992). Ions, primarily Na^+ and Cl^-, gained after ingesting the medium and food items and via gill diffusion, are excreted at the gill by chloride cells (Hirano and Mayer-Gostan, 1976). Diffusional H_2O loss from the gills is counteracted by uptake in the intestine by enterocytes (Rawdon and Cornish, 1973; Viellette, 2004). Teleost osmoregulation in SW is shown schematically in Figure 2.2.

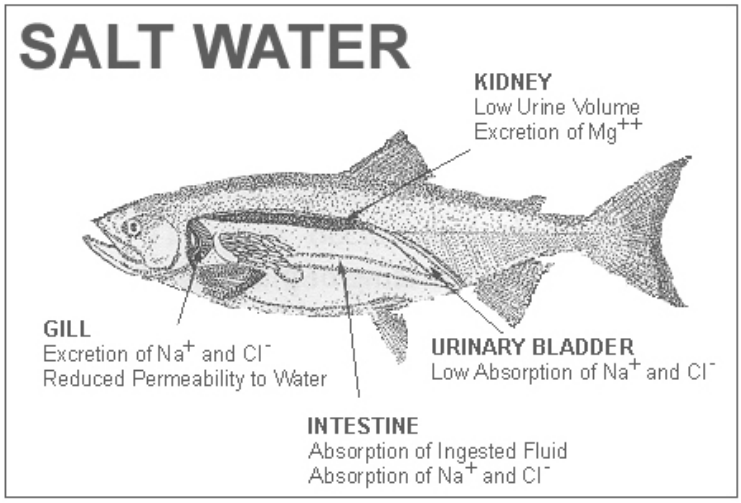

Figure 2.2. Osmoregulation in a SW Salmonid (ocean-type Chinook) (modified from Healey, 1991).

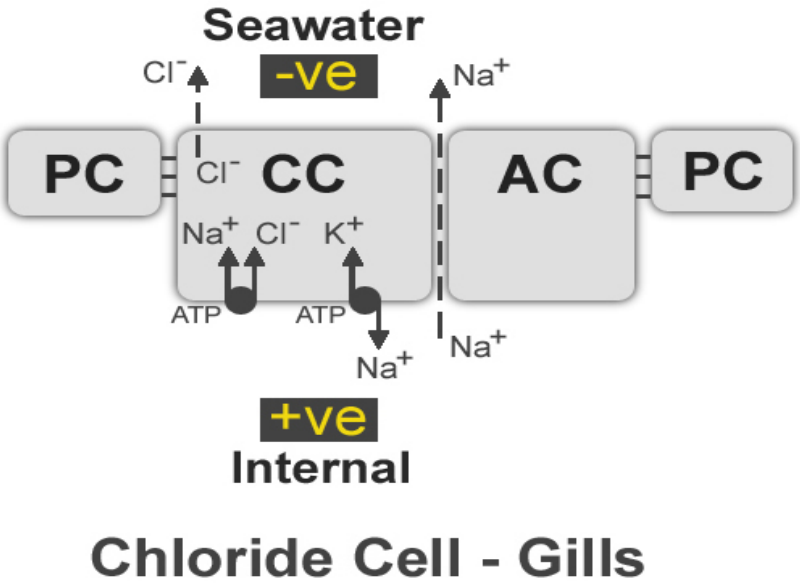

Figure 2.3. Schematic model of a chloride cell (CC) of a seawater adapted teleost fish gill showing its association with the accessory cells (AC) and epithelial pavement cells (PC). Cl^- and Na^+ movement pathways are indicated by dashed lines, through chloride channels and between chloride and accessory cells respectively. Basal active transport of Cl^- and Na^+ is occurring. A basal Na^+/K^+-ATPase drives a negative outward gradient. Peak transepithelial flow is across chloride cells. (Based on Withers, (1992)).

Smoltification in salmonids involves an increase in chloride cells in the gills (Usher, 1988). A greater abundance of chloride cells is accompanied by increased infolding of mitochondrial membranes and mitochondrial populations within cells (Withers, 1992). Concomitant with this, apical crypts and accessory cells develop around the chloride cells (Withers, 1992). Figure 2.3 is a schematic of chloride and associated cells in the SW acclimated teleost gill and shows active ion excretion. SW acclimation is primarily regulated via cortisol, which is released in response to environmental salinity changes (Matty, 1985; Viellette, 2004). FW acclimation from SW results in these processes being reversed, driven by prolactin (Matty, 1985; McCormick, 2001).

Cortisol has been shown to differentiate chloride cells and associated cellular machinery in the teleostean gill (Matty, 1985), and Na^+/K^+-ATPase activity in both the gill and intestine, primarily in the pyloric caecae of the intestine in salmonids (Shehadeh and Gordon, 1969; Viellette, 2004). The basis of intestinal ionic uptake and ion-associated water uptake is shown schematically in Figure 2.4. This Figure indicates the processes occurring in a SW acclimated teleost intestinal/pyloric caecal enterocyte. Both *in vitro* (Oide and Utide, 1967; 1968 Shehadeh and Gordon, 1969) and *in vivo* (Viellette, 2004) studies have shown these processes to occur in SW teleost intestine.

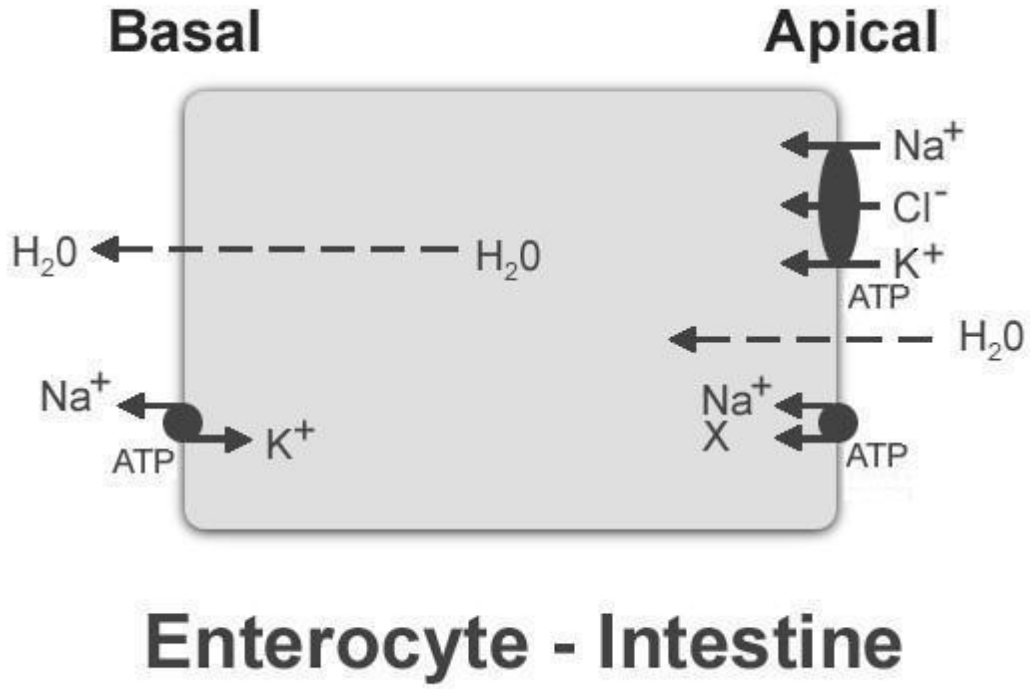

Figure 2.4. Schematic model of Na^+, K^+ and Cl^- transport across an enterocyte of the SW adapted teleost fish. H_2O movement is indicated by the dashed lines. Water movement is across transmembrane aquaporins at both the apical and basal surfaces of the enterocyte (Agre *et al.* 1998; Aoki *et al*. 2003). Water uptake from intestinal lumen (apical) to plasma (basal) is driven by a salt gradient created by Na^+/K^+-ATPases in the basolateral membrane. Na^+ entry across the apical boarder is via Na^+-K^+-$2Cl^-$ co-transporters and via coupled amino acid or sugar (X) transport. Permeable tight junctions are also thought to provide a passage for water movement (Wilson *et al*. 2005).

Viellette (2004) has found that in Chinook salmon the pyloric caecae of the proximal intestine account for up to 70% of the nominal area of the intestine and showed their role in ion-linked fluid uptake. Therefore, serum osmolality and direct volumetric rates of isolated pyloric caecae transport were determined in GDAS +ve and –ve fish. This was done in order to characterise changes in homeostatic ability of pathologically affected fish.

As the literature suggests, drinking in SW adapted teleosts is constant and copious (Oide and Utide, 1967; Usher, 1998; Withers, 1992). For the intestinal brake hypothesis (Chapter 1) to be a valid mechanism for GDAS development, constant and copious drinking must occur in conjunction with the ingestion of low cohesion feed. Therefore it was hypothesised that GDAS would develop in SW, when a low cohesion diet was fed. A SW trial was planned feeding diets of low and high cohesion in order to test this hypothesis.

2.2.4 Gastric Evacuation

Gastric evacuation is the movement of stomach digesta into the intestines for nutritive uptake (Weisbrodt, 1991). The gastric component of the emptying process is the result of the activity of three layers of smooth muscle cells; an outer longitudinal layer, a middle circular layer and an inner oblique layer (Weisbrodt, 1991). The circular layer is the most prominent and is present in most areas of the GI tract (Weisbrodt, 1991). The rate of gastric evacuation is the rate at which chyme is released from the distal part of the stomach (pyloric stomach) into the proximal intestine and pyloric caecae and in vertebrates is regulated by the pyloric sphincter (Heading, 1980). Gut motility in non-mammalian vertebrates, as in mammals, is controlled by the presence of food, by autonomic nerves and by hormones (Olsson and Holmgren, 2001). Feeding and the presence of food initiates contractions of the stomach wall and subsequently gastric emptying by peristalsis, migrating motor complexes (MMC's) and other patterns of motility (Weinbeck, 1998; Olsson and Holmgren, 2001). Gastric secretions are also produced in response to nutritive input and gastric distension; these secretions include

enzymes (e.g. pepsinogens) and acids (e.g. hydrochloric acid) (Heading, 1980; Withers, 1992).

The properties of the food item consumed during feeding may have profound effects on the nature of gut motility in vertebrates (dos Santos and Jobling, 1988). For example, Jobling (1987) has shown that particle size and dietary energy content affects gastric evacuation in fish. Several other researchers have shown that liquid meals lacking in nutrients are evacuated in an exponential fashion while homogeneous, nutrient rich meals are emptied in a more linear manner, with the degree of slowing of gastric emptying being directly related to caloric value of the meal (Jobling, 1987). Non-nutrient bulk is emptied rapidly from the stomach and inert solids and indigestible foods may be passed as relatively large particles (Jobling, 1986). Liquid meals are emptied from the stomach much more rapidly than digestible solids, even when the liquids are rich in nutrients (Jobling, 1986). Non-nutritive bulk and liquid meals are emptied rapidly (Jobling, 1986).

It has been shown in a number of vertebrate groups that the pattern of gastric evacuation is influenced by feedback signals from various receptors located in the upper intestinal tract and by factors affecting the rate of physical and chemical breakdown of the food particles (Jobling, 1987). Ideally, food is emptied from the stomach into the intestine in boluses, at a rate that ensures that the secretory and absorptive capacities of the intestine are not exceeded (Liddle, 1986). This process has been characterised in mammals (ileal brake) and fish (intestinal brake) (Chapter 1). There is an extensive body of literature that suggests almost anything can affect the rate of gastric emptying (Jobling 1987; dos Santos and Jobling 1988; Olsson and Holmgren, 2001). The most important

factors include stomach fullness, frequency of feeding, and the composition of the chyme in the stomach and intestine (solid or liquid, osmolarity, carbohydrates, fat, protein etc) which affect the tonus of the pyloric sphincter and the tonus of the stomach wall (Olsson and Holmgren, 2001).

For example, the amino acid profile has a slight effect on the rate of emptying in salmonids. Storebakken *et al*. (1999) estimated gastrointestinal evacuation of Atlantic salmon using yttrium and ytterbium oxides ($Y_2 O_3$ and $Yb_2 O_3$) and collection of faeces from the outlet water of the experimental holding tanks. The fish were fed three diets with different protein sources: fish meal (FM) (55% FM); soybean meal (SBM) (35% FM and 31% SBM); and bacterial meal (BM) (34% FM and 20% BM). Rate of passage was estimated by first feeding the fish the diets labelled with the inert marker $Y_2 O_3$ and then changing to the same diets labelled with $Yb_2 O_3$. Faeces were obtained by sieving of the outlet water and were pooled by tank every 3 h for 81 h after the switch in diets. The evacuation of the diets was expressed as the ratio between $Yb_2 O_3$ and the sum of inert markers ($Y_2 O_3$ + $Yb_2 O_3$) in the faeces. The estimated rates of passage were described by sigmoidal curves (R^2=0.98). The time for $Yb_2 O_3$ to reach 50% of maximum marker concentration was ~18h in FM and BM meals and 20h in SBM fed fish. The amino acid composition of the diets resulting in the slowest rates of evacuation may have had relatively higher tryptophan levels than other diets.

Similarly, Tekinay and Davies (2002) studied the effects of the dietary carbohydrate level on gastric evacuation and return of appetite in the rainbow trout. Three experimental diets containing 14.9% (low carbohydrate; LC), 31.4% (medium carbohydrate; MC) and 42.3% (high carbohydrate; HC) extruded wheat meal were fed to

rainbow trout to investigate the significance of carbohydrate level on the rates of gastric evacuation and return of appetite ('feed intake'). Gastric evacuation measurements were performed by serial slaughter and recovery of digesta whilst X-radiography was used to estimate the rates of return of appetite. The gastric evacuation rate of the LC group was significantly different from that of the MC and HC treatments, while there was no significant difference between the emptying rates of the MC and HC groups. LC diets were evacuated faster than MC and HC diets.

All these data suggest that a fine particulate, high-nutrient pelleted meal of low cohesion would rapidly disaggregate in the stomach and could therefore potentially result in a different pattern of evacuation than a feed pellet meal with the high cohesion. This could be more pronounced when accompanied by substantial drinking of the medium, (i.e. in SW), as chyme formation may be accelerated. This is hypothesised to result in a high nutrient chyme passing into the proximal intestine activating a similar mechanism to the ileal brake, the intestinal brake (Olsson and Holmgren, 2001). Therefore, it was hypothesised that a low cohesion feed would result in a different pattern of gastric evacuation in SW smolt both affected and unaffected by GDAS. This is consistent with the findings of Jobling (1986) and is consistent with the intestinal brake mechanism. Consequently, an investigation of gastric evacuation of digesta in fish fed diets of low and high cohesiveness in SW was planned.

2.3 Materials and Methods

2.3.1 FW Trial

FW raised smolt were transferred at NZKS's Tentburn hatchery in Canterbury, New Zealand from commercial raceways into two prepared raceways fed with high quality bore water. A total of 1000 fish (mean mass: 71.3g ± 14.1g, fork length: 185 ± 23mm, and condition factor (CF) 1.12 ± 0.19 (± SEM)) were transferred into two raceways (i.e. 500 fish in each). Each group was fed one of two diets (diet A or diet B) over a 6-week period. Randomly selected fish were removed with a 'dip net' at weekly intervals for assessment and experimental tissue extraction and analysis. Upon removal, the utmost care was taken to minimise stress in the animals. The fish were then transported in aerated ice-chilled fish boxes to the Physiology Laboratory in the School of Biological Sciences, at the University of Canterbury. Upon arrival, the fish were stunned by hitting the dosal side of the head with a blunt object. The gut was then dissected out and the stomach and intestine isolated. Following the removal of the surrounding connective tissue, stomach and intestinal tissues were cut into rings and placed into Freshwater Salmon Ringer (FSR). FSR (NaCl 136.89mM; KCl 2.11mM; $MgCl_2.6H_2O$ 0.99mM; $CaCl_2.2H_2O$ 1.30mM; $C_6H_{12}O_6$ 10.00mM; BES 0.99mM; Na^+-glutamate 0.30mM; L-glutamate 0.40mM; Na^+-asparate 0.02mM; DL-carnitine 0.05mM) which had a mean ± SEM osmolarity of 299 ± 2 mOsmol.L^{-1} (adapted from Janssen, 2003) . Tissue samples were stored in FSR at 10°C until needed.

2.3.2 SW Trial

FW raised smolts were transferred from NZKS's Takaka hatchery (Golden Bay) to a FW holding raceway at Kaituna (Blenheim). There they were fed until the mean population weight was approximately 70g. A 13000*l* experimental SW recirculation system comprising two header tanks, eight 1000*l* holding tanks, two 1000*l* waste collection tanks, biofilter, carbon filter, sand filter and dacron (particulate) filter was utilised as a holding system for the duration of the trial. Tanks were cleaned twice daily. Water chemistry was monitored and adjusted as necessary. Random numbers were generated and groups of 20 or 30 hand caught fish were allocated to the experimental tanks as per the random number schedule until all tanks were filled with 80 fish (i.e. 8 tanks with 80 fish in each), (mean mass: 75.8g ± 12.5g, fork length: 190 ± 40mm, and condition factor (CF) 1.22 ± 0.39 (± SEM)). Each of the 8 tanks were then randomly assigned to one of two diets (A or B), amounting to 4 tanks each being supplied with one of the two diets. Fish were fed twice daily for 5 weeks until cessation of feeding (satiation). Randomly selected fish were removed by dip netting, at weekly intervals for assessment and experimental tissue extraction and analysis. Again, the tank selected for sampling was determined via a random number generator. Fish were killed on site by a blow to the dorsal side of the head.and taken to the lab, which was only a short walk from the holding tanks. Following the removal of the surrounding connective tissue, stomach and intestinal tissues were then cut into rings and placed into Saltwater Salmon Ringer (SSR), which was FSR with NaCl molarity increased to 159.60mM. The Ringer had a mean ± SEM osmolarity of 340 ± 5 $mOsmol.L^{-1}$. Tissue samples were stored in SSR at 10°C until needed.

2.3.3 Fish for Other Experiments

Fish for all other experiments were obtained from either NIWA's Silverstream Hatchery (Canterbury) or Isaac's Salmon Farm (Canterbury). These fish were transported in aerated ice-chilled fish boxes to the Physiology Laboratory in the School of Biological Sciences, at the University of Canterbury. Upon arrival, the fish were either killed by stunning or kept in the University aquarium.

2.3.4 Diets

Diet A and Diet B are both commercially manufactured diets. Diet A is a steam pelleted diet of fine particle size (5-10 μm) that rapidly disaggregates in the stomach (NZKS; unpublished data) and when subjected to NZKS's pellet cohesion test. The test involves pellet saturation and agitation. The test protocol has been omitted from the methods of this thesis due to an intellectual property agreement between the author and NZKS. The diet showed ~1-2% 'retention' after a 24-hour period of treatment and subsequent sieving through 2.25 aperture sieve. Diet B is an extruded ('cooked') diet also of fine particle size (5-10 μm) that is not easily disaggregated in the stomach (NZKS; unpublished data) and when subjected to the pellet cohesion test shows a ~70 % 'retention' after a 24-hour period of treatment and subsequent sieving through 2.25 aperture sieve. A Proximal analysis of the two diets showed almost identical nutritional constituents (NZKS; pers. com.). Therefore pelleting was the primary determinant of physical cohesion.

2.3.5 GDAS Assessment

As detailed above, fish used for experiments were randomly removed weekly (n=50). These fish were deemed to be GDAS +ve or GDAS –ve upon sacrifice. When GDAS was occurring in an experimental population, only GDAS +ve fish were used for comparison with the control groups (i.e. diet B in FW and SW). If a fish displayed one or more of the following symptoms they were deemed to be GDAS +ve :

1. **Chyme-filled distended stomach with reduced rugal fold thickness.**
2. **Fluid/solids present in the swimbladder.**

2.3.6 Myography

Isolated cardiac stomach and pyloric sphincter rings were pharmacologically challenged with ACh and KCl to induce spontaneous contractions and completely depolarise smooth muscle cell populations, respectively. In doing so, the contractile response of stomach circular smooth muscle could be characterised. Fish were killed by stunning and subsequently incised along the ventral side of the abdomen from the gills to the anus. The whole GI tract was then dissected out and the stomach and intestine were isolated. Cardiac stomach rings were cut from the cardiac stomach ~10mm from the end of the oesophageal sphincter. Pyloric sphincters were removed at the junction between the stomach and intestine and cut in half. These were stored in FSR or SSR until needed for myography. Cardiac stomach rings and sphincters were 'strung-up' between a fixed point and a calibrated Powerlab™ pressure transducer, using surgical stitching silk. The preparation was bathed in 20ml of aerated salmon Ringer. Following preliminary testing,

tissues were tensioned in the optimum range (so as not to under-tension or damage the tissue) and allowed to rest before ACh or KCl was added to the Ringer baths. Transducer output was fed directly into a HP Compaq nx 9040 laptop running Microsoft Chart 5 ™, where an instantaneous readout was recorded to disc. Contraction force was recorded in milligrams (mg). The stomach tissue was challenged by ACh ($1x10^{-4}$ M and $1x10^{-3}$ M) in a cumulative way and then finally by sufficient KCl (125mM) to depolarise the muscle cells. Additions were made 15 minutes apart. After the experiment, all tissue samples were blotted dry and weighed to 4dp. All data were normalised using wet tissue mass.

2.3.7 Fluid Transport by Pyloric Caecae

A method first used by Viellette (2004) was adapted to quantify the active movement of water and dissolved ions in the pyloric caecae from the mucosal surface to the serosal surface. FSR or SSR was exposed to both surfaces in FW and SW respectively. Since there was no osmotic gradient between the surfaces, any movement between the two represented an active movement. Fish were incised and dissected as described in the myography experiments. Isolated individual caecae (n=5 per fish) were cut at their base and gently flushed once with Ringer solution. As reported by Veillette, (2004) only one flush is necessary. Polythene tubing (cannula) was pre-filled with Ringer and subsequently inserted and the caecae tightly secured with stitching silk. Gravity was determined not to have an influence on caecal water movement as there was a linear pattern of fluid movement. If there was a gravitational effect, the pattern of movement would be non-linear. The non-everted caecae-cannula preparation was mounted upright in aerated Ringer at constant temperature (10°C). Height of the fluid (meniscus) in

millimetres (mm) was measured. After a 30 minute settling period, recordings were taken every 15 minutes for 2 hours. Through weight assessment, the cannula used was determined to hold 60n*l* of fluid in each mm. After the experiment, all tissue samples were blotted dry and weighed to 4dp. The data was normalised using wet tissue mass. Subsequently, the rate of movement over time was calculated from linear regression lines.

2.3.8 Serum Osmolality

Blood samples were collected immediately after death via caudal puncture. A sterile insulin syringe was inserted immediately proximal to the anal fin and between 0.5 and 1 ml of blood was withdrawn. The blood was then transferred to a 1.5ml Eppendorf tube for clotting (10 min) and spinning for 10 mins at 10,000 RPM in an Eppendorf ® centrifuge. Serum osmolality was then determined using a calibrated Wescor® vapour pressure osmometer (Model 5100C). Samples were not pooled.

2.3.9 Histology

Cardiac stomach samples were collected and preserved in 10% phosphate buffered formalin (PBF). These samples were then subjected to a dehydration series and embedded in Ralwax. The embedded tissue samples were thin sectioned on a microtome (at 5 and 7 μm), mounted and stained utilising Mayer's double strength haemalum and eosin Y for microscopic examination. All of the sections were prepared by hand. For detailed methods please refer to the appendix (p149).

2.3.10 Vital Statistics

Weight, fork length and scale loss (scored as 0-3; 0=zero scale loss and 3=extreme scale loss) were recorded for each fish killed. Weight and fork length were used to calculate condition factor (CF). CF was calculated by the equation:

$$\mathbf{K = 100,\,000}\ W / L^3$$

where:
K = The coefficient of condition
W = the weight of the fish in grams
L = the fork-length of the fish in millimetres

Each fish killed was assessed for the presence of GDAS symptoms and adjudged to be either GDAS +ve or –ve.

2.3.11 Estimation of Gastric Evacution

The diets were analysed for water content (%) by dividing the dry mass by the wet mass. This was done by weighing and subsequently drying the feed pellet samples at a constant temperature (80°C) for 24 hours. This percentage was used to correct for stomach content dry mass after known times after ingestion. SW acclimated Chinook smolt (2 weeks) were force-fed a known amount of the test diet (~1g) via a modified insulin syringe fitted with silicon tubing. The fish were fin clipped in order to correlate individual fish with meal weight after sacrifice, and separated into holding tanks based on diet. Experiments were conducted at time intervals of 4, 12 and 24 hours after feeding. As previously, experimental fish were killed by stunning. After dissection, the stomach content was removed by cutting the stomach along its entire length and scraping out the contents into a foil ‘pie tray’ of known weight. The trays, containing the stomach content samples were

then weighed. The trays were then placed in an oven at 80°C for 24 hours, and weighed immediately after coming out of the oven.

2.3.12 Data Analysis

Prior to analysis, all data were examined for normality using normality plots in Microsoft Minitab® and determined to have Gaussian distributions. Diet cohesion, myography, histology and vital statistics data in FW and SW were compared using unpaired Student's t-tests. Linear regression and subsequently, F-tests on the slopes of the linear regression lines were utilised to test for differences in rate of fluid transport in FW and SW. In SW, 2^{nd} order polynomial regression characterised the serum osmolality best. Differences in these data were tested by unpaired Student's t-tests. Analysis was performed on Microsoft Chart 5®; Excel; Graphpad Prism 4® and Graphpad Instat 3®.

2.3.13 Animal Ethics

Animals were killed by stunning rather than anaesthesia, due to the known relaxant effect of anaesthetic on the smooth muscle of experimental fish (Hill *et al.* 2004). All experiments conducted were approved by the University of Canterbury Animal Ethics Committee.

2.4 Results

2.4.1 Freshwater

In the FW trial, GDAS was not induced by the feeding of either diet (Table 2.1 and Figure 2.5). When SL and CF were analysed using linear regression there was no significant difference between diets A vs B) (CF P=0.0788, SL P=0.215). The slopes of the linear regression lines of serum osmolality (Figure 2.6) did not differ significantly (P=0.9020) when compared using an F-test. Therefore, the data were averaged and this value used to quantify the change in serum osmolality in SW adapting smolt fed the two different diets in the SW trial. Tissue mass standardised (TMS) caecal Ringer movement (mucosal to serosal) in FW was analysed using linear regression. Comparisons were made on the slopes of the two diet treatments using an F-test within weeks. No significant difference was detected between groups in any of the weeks throughout the trial (Figure 2.7). Rate of movement was calculated from the slope of these data, which ranged from 4.46 ± 0.07 to 7.27 ± 0.04 n*l*.min^{-1} (Table 2.2). TMS pyloric sphincter (KCl, Figure 2.8) and cardiac stomach (ACh 1x10^{-4} M, Figure 2.9; ACh 1x10^{-3} M, Figure 2.10; and KCl, Figure 2.11)) contractility were compared by unpaired Student's t-tests on a weekly basis based on diet. There was no significant difference between diet treatments in any week. Exposure to 125mM KCl produced a larger contraction than either ACh treatment (c.30%).

Figure 2.5. GDAS –ve fish in FW, showing the GI tract.

Table 2.1. Summary table of GDAS incidence (% GDAS), mean ± SEM serum osmolality (SO), scale loss (SL) and condition factor (CF) of Chinook smolt fed either diet A (low cohesion) or diet B (control/high cohesion) in FW. n=4, except in weeks 4, 5 and 6 where n=8 for each diet/week combination.

FW Diet B				
Week	**% GDAS**	**SO**	**CF**	**SL**
1	0	295 ± 1	1.28 ± 0.02	0.11 ± 0.03
2	0	294 ± 3	1.35 ± 0.03	0.13 ± 0.02
3	0	296 ± 2	1.35 ± 0.06	0.23 ± 0.02
4	0	298 ± 3	1.34 ± 0.05	0.25 ± 0.01
5	0	291 ± 4	1.38 ± 0.03	0.33 ± 0.04
6	0	290 ± 3	1.34 ± 0.02	0.45 ± 0.03
FW Diet A				
Week	**% GDAS**	**SO**	**CF**	**SL**
1	0	293 ± 2	1.34 ± 0.03	0.14 ± 0.04
2	0	300 ± 1	1.37 ± 0.04	0.50 ± 0.02
3	0	295 ± 1	1.32 ± 0.02	0.63 ± 0.04
4	0	292 ± 2	1.25 ± 0.05	1.17 ± 0.05
5	0	295 ± 2	1.32 ± 0.03	1.25 ± 0.03
6	0	295 ± 1	1.30 ± 0.03	0.75 ± 0.02

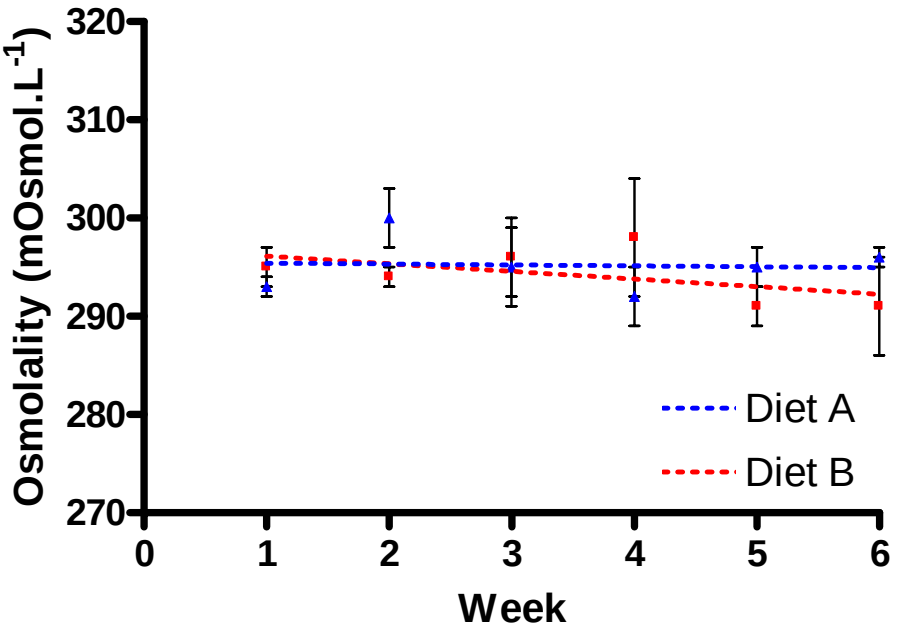

Figure 2.6. Serum osmolality (mOsmol.L^{-1}) in Chinook smolt in FW fed either diet A (n=36) (low-cohesion, blue line) or diet B (n=36) (high-cohesion, red line) over a 6-week period. Linear regression lines were fitted to the data (Diet A R^2=0.45, equation y = -0.1581x + 295.69; Diet B R^2= 0.56 y =-0.6922x + 296.5. The regression line slopes were not significantly different from each other (P=0.9020) when compared by an F-test. Data are mean ± SEM.

Table 2.2. Summary table of mean ± SEM caecal Ringer transport rates (RTR) (*nl*.g^{-1}.min^{-1}) in FW Chinook smolt. There was no difference in slope of the linear regression lines when compared by F-tests. The R^2 and P values are summarised. n=4, except in weeks 4, 5 and 6 where n=8 for each diet/week combination.

FW	Diet	RTR (*nl*.g^{-1}.min^{-1})	R^2	P value
WK1	Diet A	5.28 ± 0.07	0.95	0.5853
	Diet B	5.53 ± 0.05	0.97	
WK2	Diet A	5.88 ± 0.09	0.98	0.0988
	Diet B	5.32 ± 0.05	0.98	
WK3	Diet A	7.27 ± 0.04	0.97	0.8550
	Diet B	5.79 ± 0.08	0.98	
WK4	Diet A	5.58 ± 0.07	0.97	0.5830
	Diet B	5.98 ± 0.04	0.98	
WK5	Diet A	5.05 ± 0.03	0.97	0.4550
	Diet B	5.31 ± 0.11	0.97	
WK6	Diet A	4.46 ± 0.07	0.98	0.0571
	Diet B	4.87 ± 0.05	0.99	

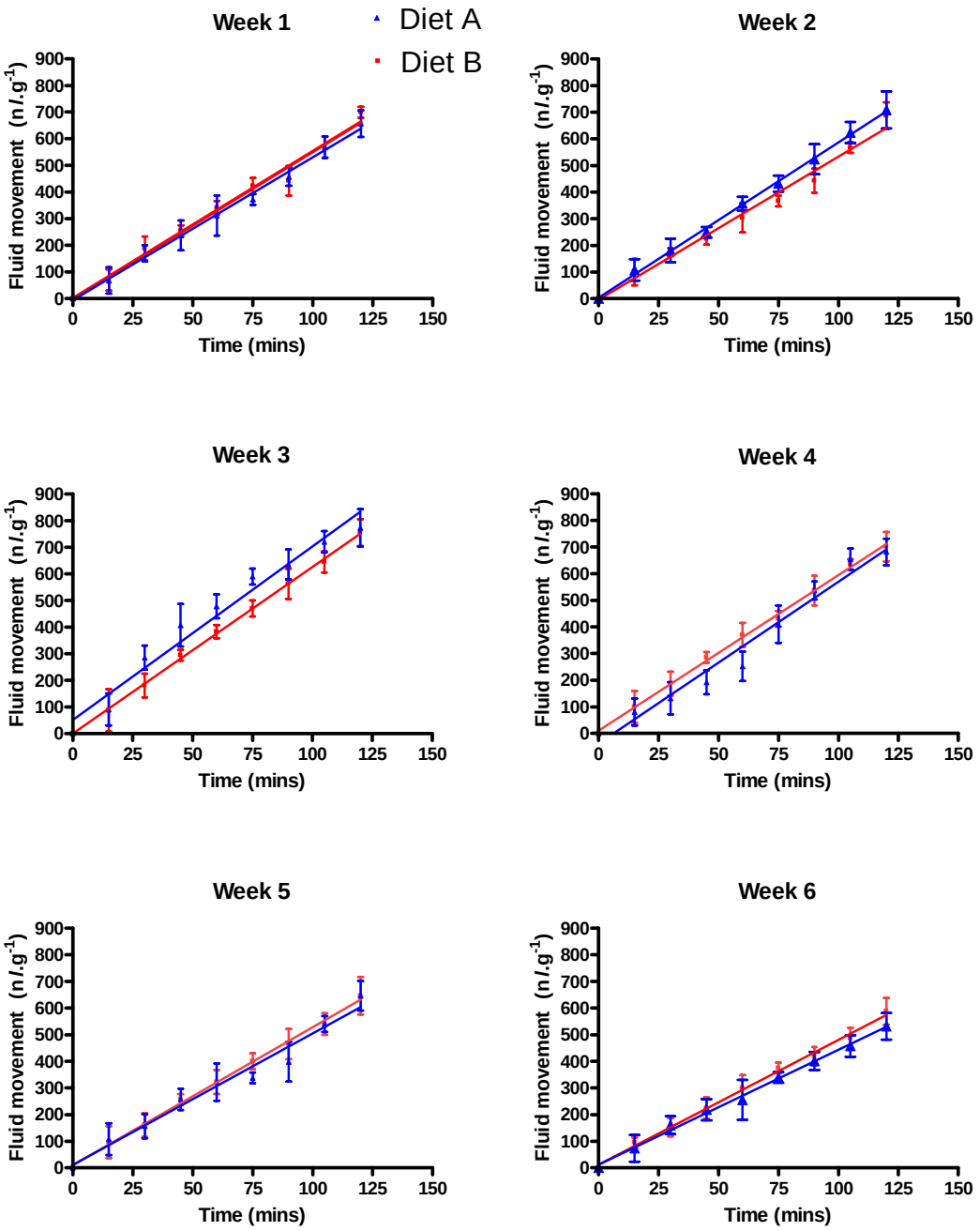

Figure 2.7. Weekly comparison of caecal Ringer transport in *nl*.g^{-1} between fish fed diet A (low cohesion, blue line) and diet B (high cohesion, red line) in FW Chinook smolt. Data are mean ± SEM. n=5 for each diet/week combination.

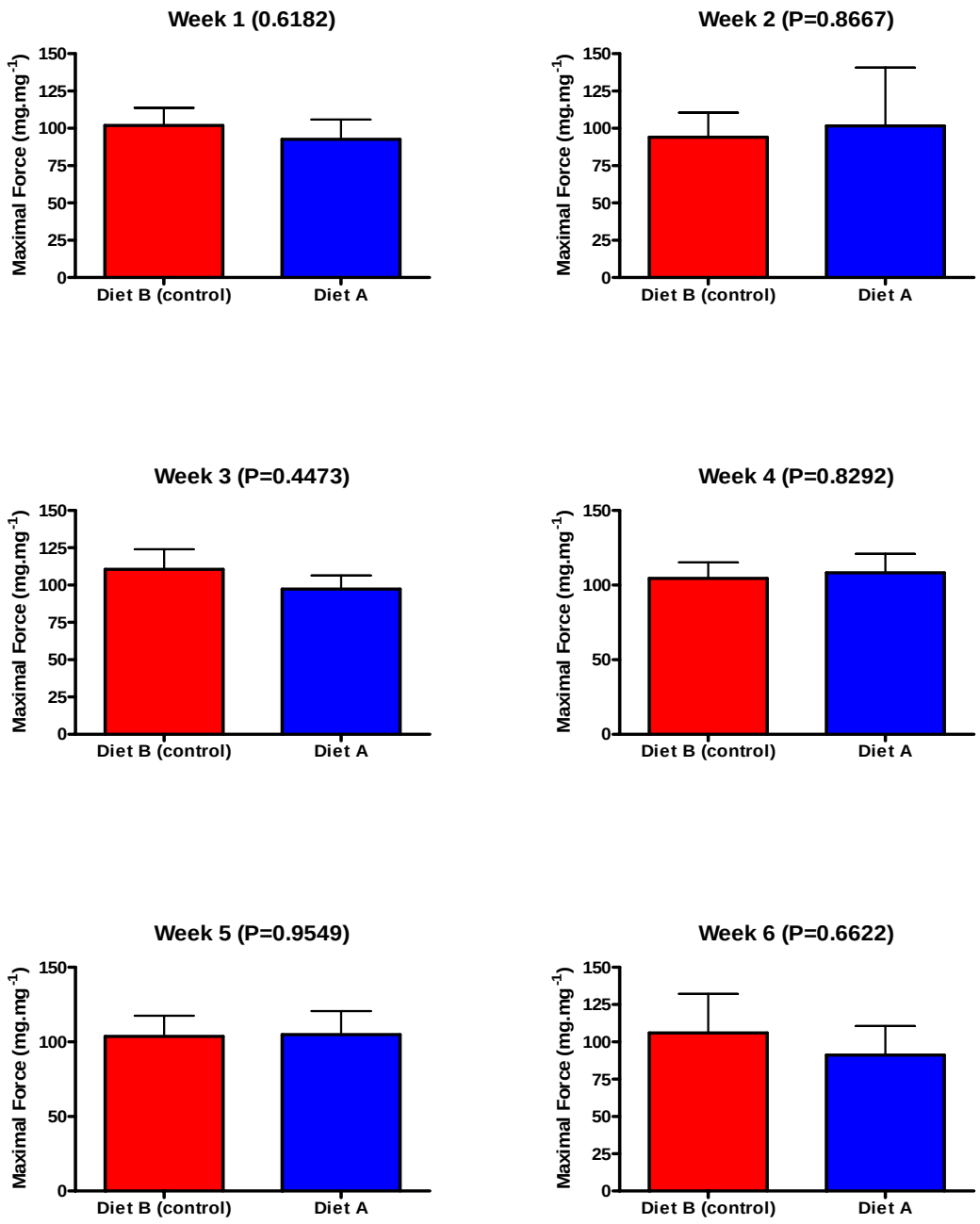

Figure 2.8. Weekly comparison of pyloric sphincter circular smooth muscle maximal contractility ($mg.mg^{-1}$) in response to KCl between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red) in FW Chinook smolt. P values of unpaired two-tailed t-tests are shown in the figure. Data are mean ± SEM. n=4, except weeks 4, 5 and 6 where n=8 for each diet/week combination.

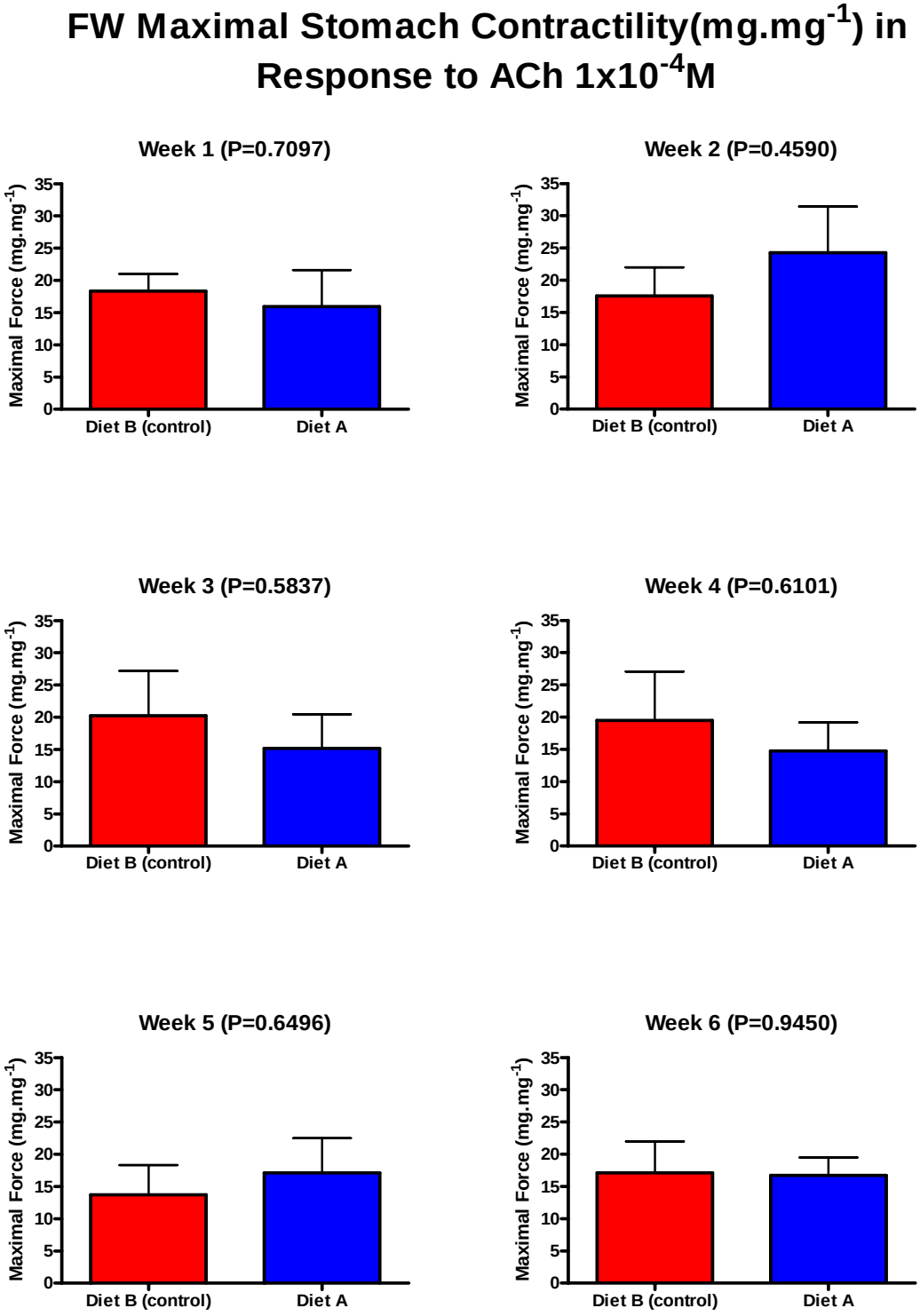

Figure 2.9. Weekly comparison of cardiac stomach circular smooth muscle contractility ($mg.mg^{-1}$) in response to ACh $1x10^{-4}$ M between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red) in FW Chinook smolt. P values of unpaired two-tailed t-tests are shown in the figure. Data are mean ± SEM. n=4, except weeks 4, 5 and 6 where n=8 for each diet/week combination.

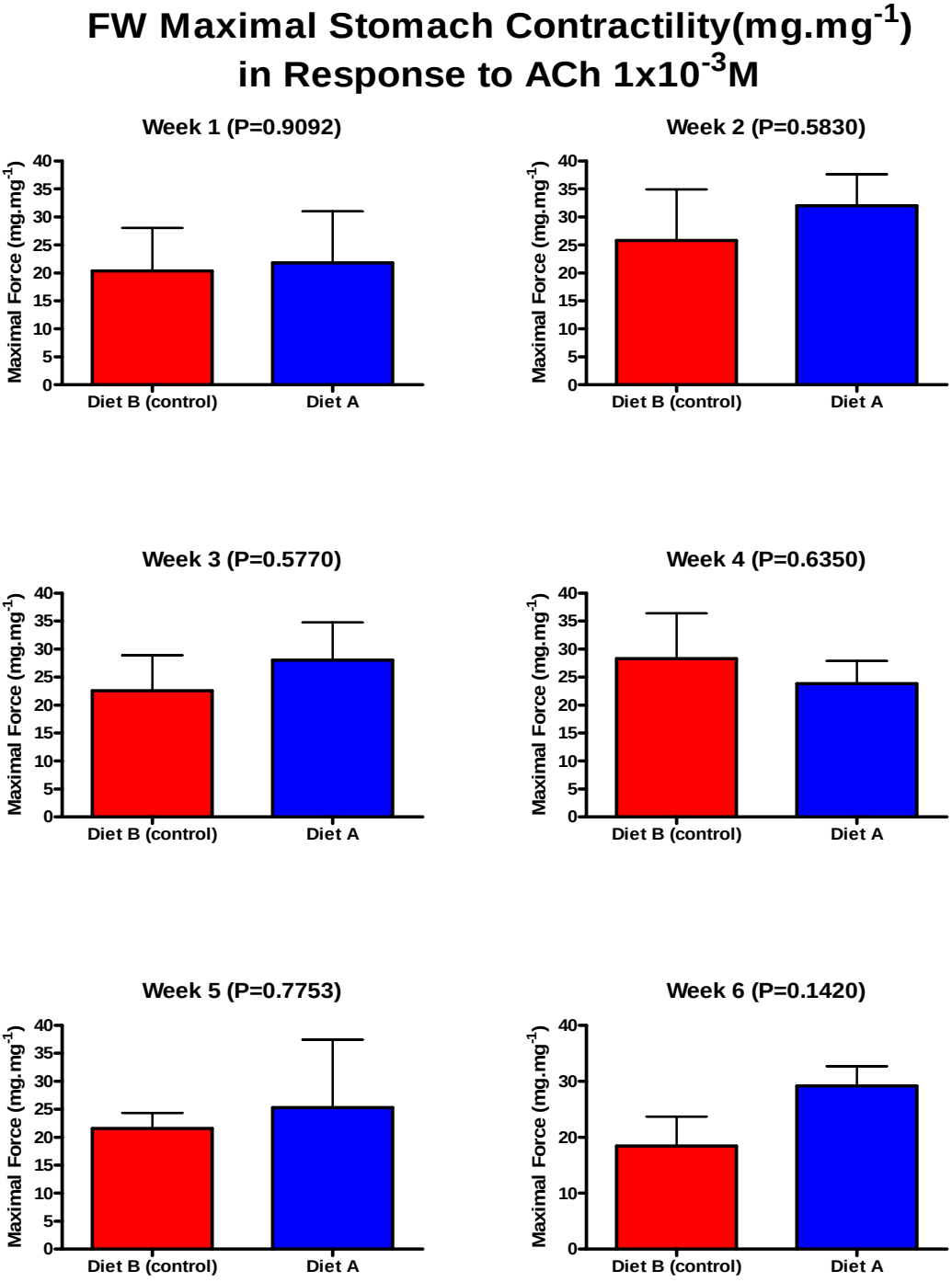

Figure 2.10. Weekly comparison of cardiac stomach circular smooth muscle contractility ($mg.mg^{-1}$) in response to ACh $1x10^{-3}$ M between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red) in FW Chinook smolt. P values of unpaired two-tailed t-tests are shown in the figure. Data are mean ± SEM. n=4, except weeks 4, 5 and 6 where n=8 for each diet/week combination.

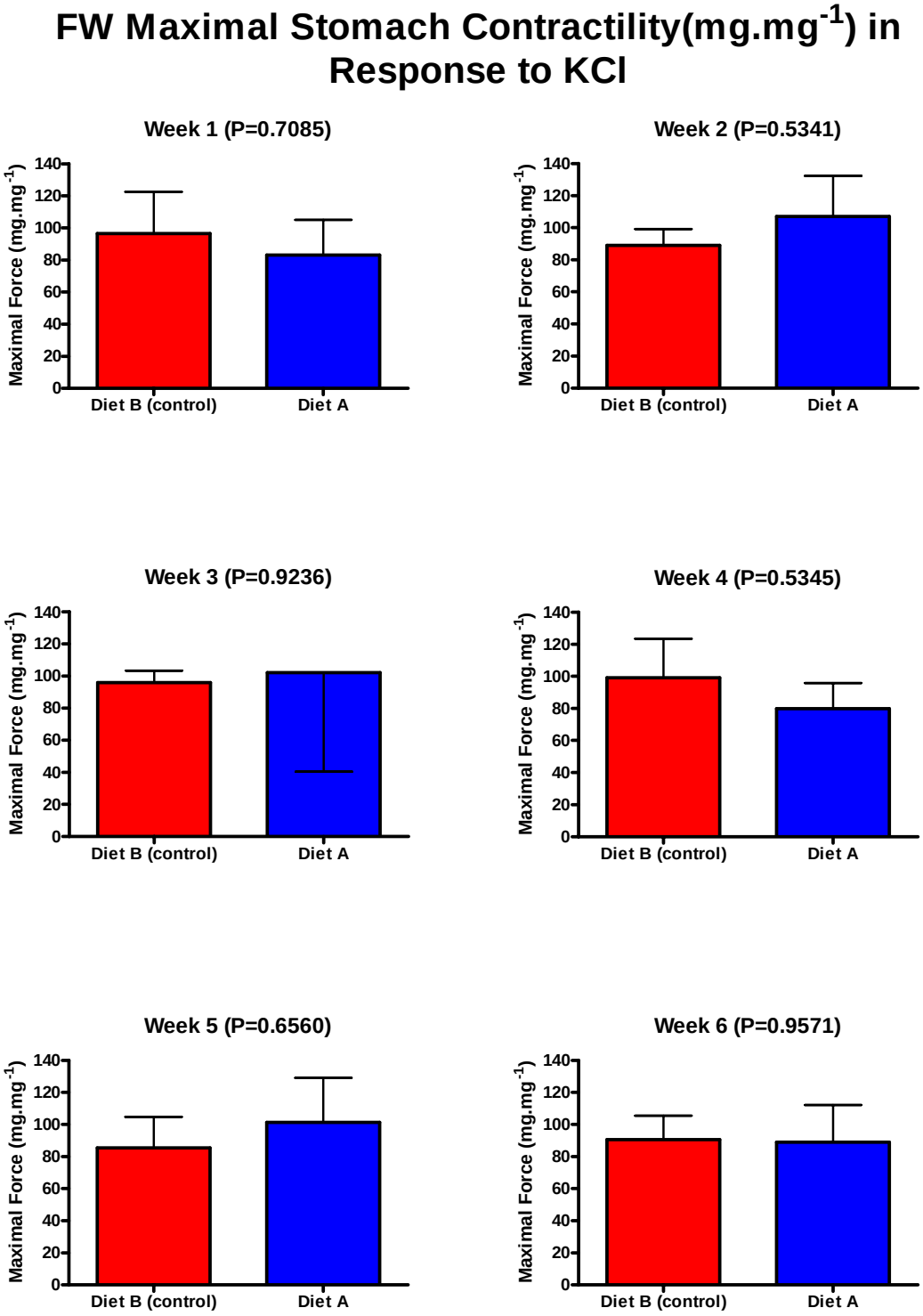

Figure 2.11. Weekly comparison of cardiac stomach circular smooth muscle maximal contractility ($mg.mg^{-1}$) in response to KCl between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red) in FW Chinook smolt. P values of unpaired two-tailed t-tests are shown in the figure. Data are mean ± SEM. n=4, except weeks 4, 5 and 6 where n=8 for each diet/week combination.

2.4.2 Saltwater

Unlike the FW trial, GDAS was induced by the feeding of diet A (low cohesion) but not diet B (high cohesion) in SW. GDAS incidence was estimated at; 0% (Week 1), 12.5% (Week 2), 55.7% (Week 3), 84.6% (Week 4) and 87.3% (Week 5) by the GDAS assessment criteria outlined in the methods. Figure 2.12 shows the dilated stomach of a GDAS +ve fish. Contrary to the data from FW, the linear regression for SL of diet A fed fish in SW had a significantly different slope to that of diet B fed fish over the 5-week period when compared by an F-test (P=0.0045) (Table 2.3). CF was not significantly different between groups (P=0.8728) (Table 2.3). Polynomial regression lines were the best fit of the osmolality data (Figure 2.13), due to the up-regulation of osmoregulatory ability associated with SW acclimation in Salmonids. Average weekly mean ± SEM serum osmolality is presented in Table 2.3. Diet A fed fish showed a higher serum osmolality over the whole 5 week trial. Serum osmolality in diet A fed fish was significantly elevated in weeks 2 (P=0.0019), 3 (P=0.0012), 4 (P<0.0001) and 5 (P=0.0024) when compared using unpaired two-tailed Student's t-tests. TMS caecal Ringer transport rate was greatly elevated in SW relative to FW in both diet treatments which ranged between 28.87 ± 0.04 and 39.74 ± 0.07 n*l*.min^{-1} (Table 2.4). When the linear regression slopes were compared between treatments by F-tests (Figure 2.14), there was a significantly reduced rate of transport in fish fed diet A in week 3 (P<0.0001), week 4 (P<0.0001) and week 5 (P<0.0001) (Table 2.4). TMS maximal pyloric sphincter circular smooth muscle contractility in response to KCl was significantly elevated in fish fed diet A relative to diet B (Figure 2.15), when compared by two-tailed unpaired Student's t-tests on a weekly basis between groups in weeks 4 (P=0.0451) and

5 (P=0.0026). It was also higher than values recorded for FW adapted fish. TMS cardiac stomach smooth muscle contractility in response to ACh $1x10^{-4}$ M (Figure 2.16) in fish fed diet A was significantly reduced relative to controls in weeks 3 (P=0.0217), 4 (P=0.0425) and 5 (P=0.0310) when compared by Student's t-tests on a weekly basis. Similarly, TMS cardiac stomach smooth muscle contractility in response to ACh $1x10^{-3}$ M (Figure 2.17) in fish fed diet A was significantly reduced relative to controls in weeks 3 (P=0.0069) and 5 (P=0.0305) when compared by unpaired two-tailed Student's t-tests on a weekly basis. In addition, TMS cardiac stomach smooth muscle maximal contractility in response to KCl (Figure 2.18) in fish fed diet A was significantly reduced relative to controls in weeks 3 (P=0.0435), 4 (P=0.0164) and 5 (P=0.0270) when compared by Student's t-tests on a weekly basis.

Figure 2.12. GDAS +ve fish in SW, showing the GI tract. Note the dilated fluid (and digesta) filled stomach. External abdominal distension was visible in this fish.

Table 2.3. Summary table of GDAS incidence (% GDAS), mean ± SEM serum osmolality (SO), scale loss (SL) and condition factor (CF) of Chinook smolt fed either diet A (low cohesion) or diet B (control/high cohesion) in SW. n=4, except in weeks 4 and 5 where n=8 for each diet week combination.

SW Diet B				
Week	**% GDAS**	**SO**	**CF**	**SL**
1	0	351 ± 7	1.32 ± 0.05	0.20 ± 0.08
2	0	350 ± 8	1.31 ± 0.03	0.13 ± 0.05
3	0	335 ± 2	1.33 ± 0.04	0.63 ± 0.04
4	0	331 ± 2	1.38 ± 0.04	0.46 ± 0.02
5	0	334 ± 3	1.47 ± 0.08	0.58 ± 0.04
SW Diet A				
Week	**% GDAS**	**SO**	**CF**	**SL**
1	0	354 ± 8	1.24 ± 0.04	0.50 ± 0.05
2	12.5	368 ± 5	1.35 ± 0.03	0.75 ± 0.02
3	55.7	354 ± 4	1.39 ± 0.02	1.50 ± 0.04
4	84.6	347 ± 2	1.43 ± 0.06	1.85 ± 0.02
5	83.3	350 ± 5	1.40 ± 0.03	2.08 ± 0.03

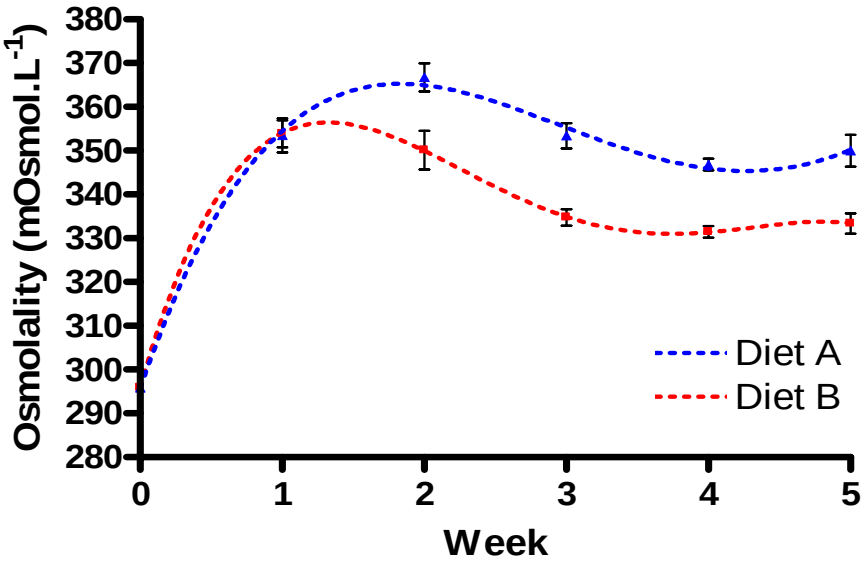

Figure 2.13. Serum osmolality (mOsmol.L^{-1}) in Chinook smolt in SW fed either diet A (n=24) (low cohesion, blue) or diet B (n=24) (high cohesion, red (control)) over a 5-week period. Fourth order polynomial regression lines were fitted to the data (Diet A R^2=0.99, equation: y= -1.187x4 + 15.631x3 - 69.642x2 + 113.32x + 295.85; Diet B R^2=0.98, equation y= -0.3075x4 + 6.5071x3 - 41.468x2 + 94.001x + 295.68. Means were significantly different from each other in weeks 2 (P= 0.0019), 3 (P=0.0012), 4 (P<0.0001) and 5 (P=0.0024) when compared by two-tailed unpaired Student's t-tests. Data are mean ± SEM.

Table 2.4. Summary table of mean ± SEM caecal Ringer transport rates ($nl.g^{-1}.min^{-1}$) in SW Chinook smolt. Linear regression lines were significantly different in weeks 3, 4 and 5 when compared by F-tests. R^2 and P values are summarised. n=4, except in weeks 4 and 5 where n=8 for each diet/week combination.

FW	Diet	RTR ($nl.g^{-1}.min^{-1}$)	R^2	P value
WK1	Diet A	30.49 ± 0.03	0.98	
	Diet B	29.82 ± 0.05	0.98	0.5714
WK2	Diet A	35.95 ± 0.08	0.99	
	Diet B	35.16 ± 0.03	0.92	0.1378
WK3	Diet A	28.99 ± 0.05	0.98	
	Diet B	38.78 ± 0.03	0.96	<0.0001
WK4	Diet A	30.31 ± 0.13	0.95	
	Diet B	39.74 ± 0.07	0.91	<0.0001
WK5	Diet A	28.87 ± 0.04	0.94	
	Diet B	39.35 ± 0.04	0.98	<0.0001

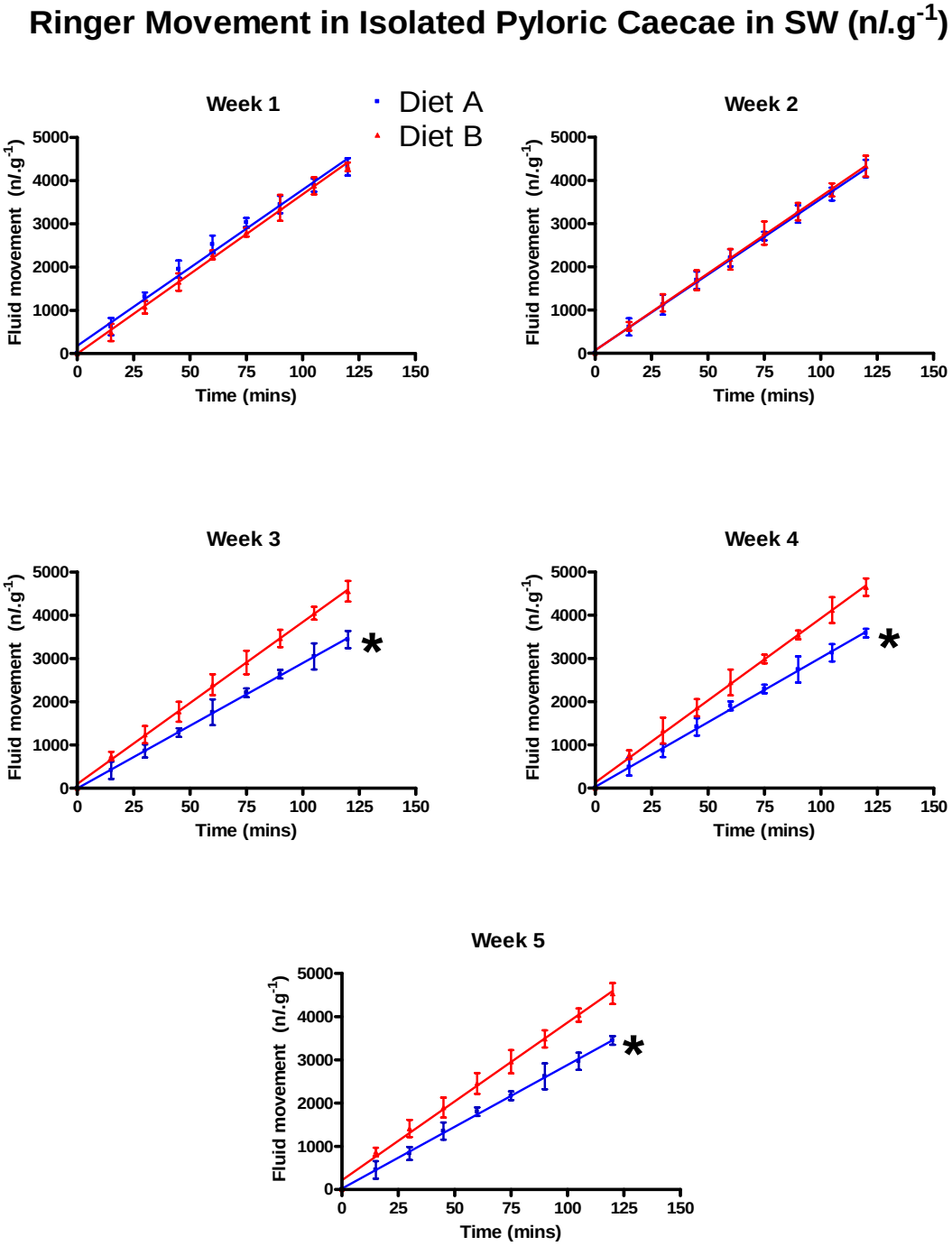

Figure 2.14. Weekly comparison of caecal Ringer transport in *nl*.g^{-1} between fish fed diet A (low cohesion, blue line) and diet B (high cohesion, red line) in SW Chinook smolt. Regression lines were significantly different in weeks 3 ($P<0.0001$), 4 ($P<0.0001$) and 5 ($P<0.0001$) Regression lines that are significantly different are marked by a *****. Data are mean ± SEM. n=5 for each diet/week combination.

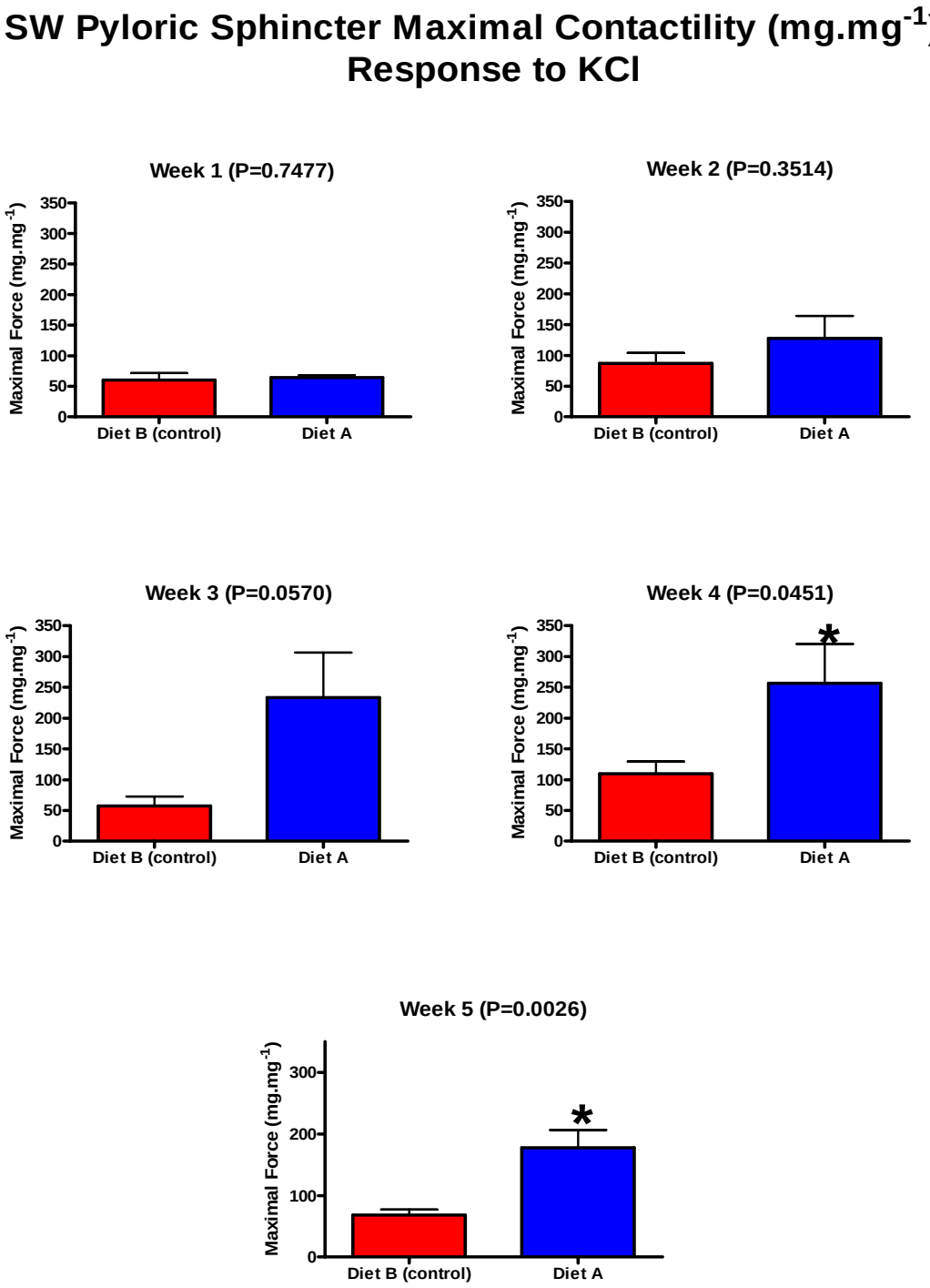

Figure 2.15. Weekly comparison of pyloric sphincter circular smooth muscle maximal contractility (mg.mg^{-1}) in response to KCl between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red) in SW Chinook smolt. Unpaired two-tailed Student's t-tests showed significant differences in weeks 4 (P=0.0451) and 5 (P=0.0026). Bars that are significantly different are marked by a *****.Data are mean ± SEM. n=4, except in weeks 4 and 5 where n=8 for each diet/week combination.

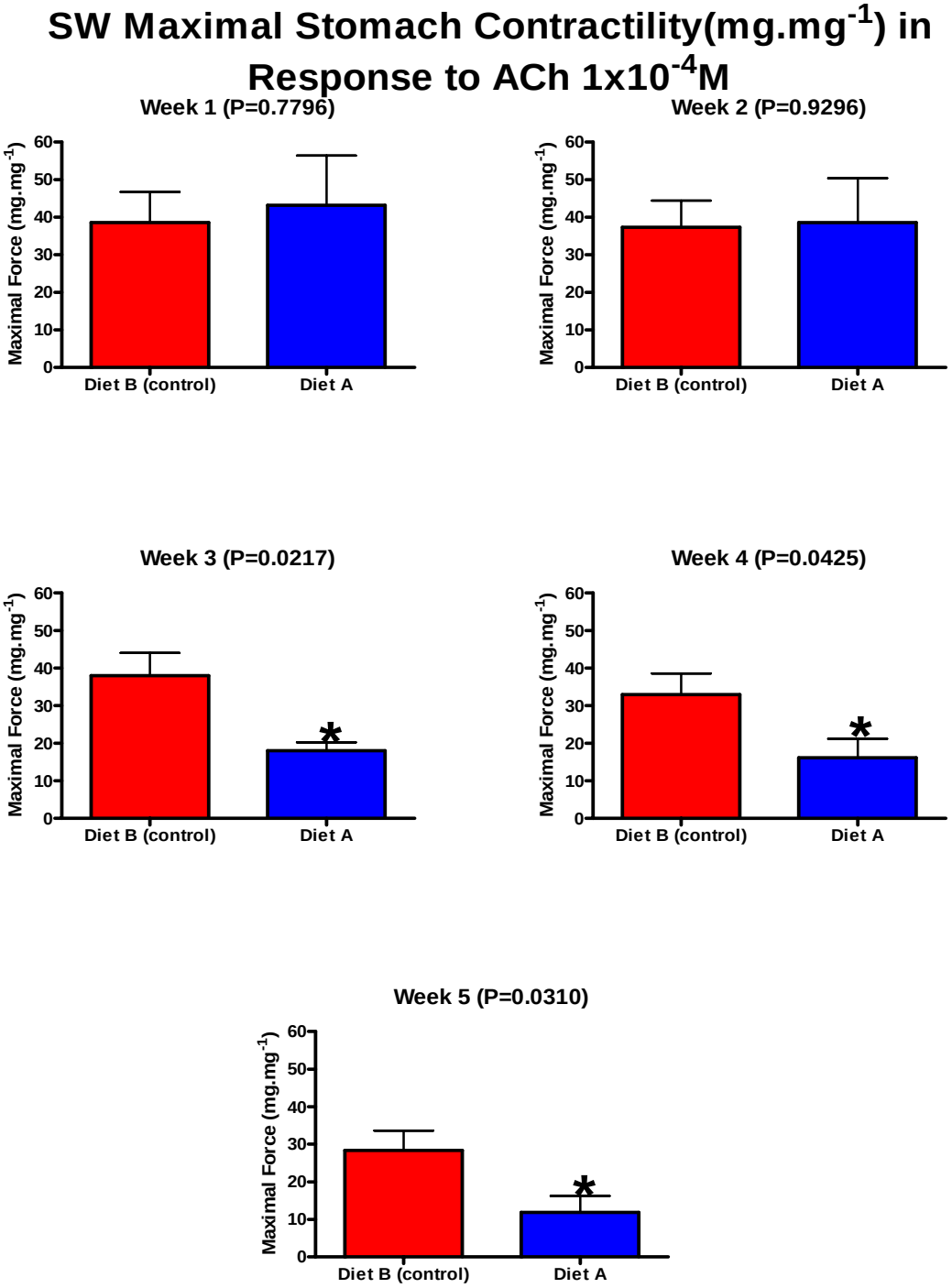

Figure 2.16. Weekly comparison of cardiac stomach circular smooth muscle contractility ($mg.mg^{-1}$) in response to ACh $1x10^{-4}$M between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red - control) in SW Chinook smolt. Unpaired two-tailed Student's t-tests showed significant differences in weeks 3 (P=0.0217), 4 (P=0.0425) and 5 (P=0.0310). Bars that are significantly different are marked by a ***.** Data are mean ± SEM. n=4, except in weeks 4 and 5 where n=8 for each diet/week combination.

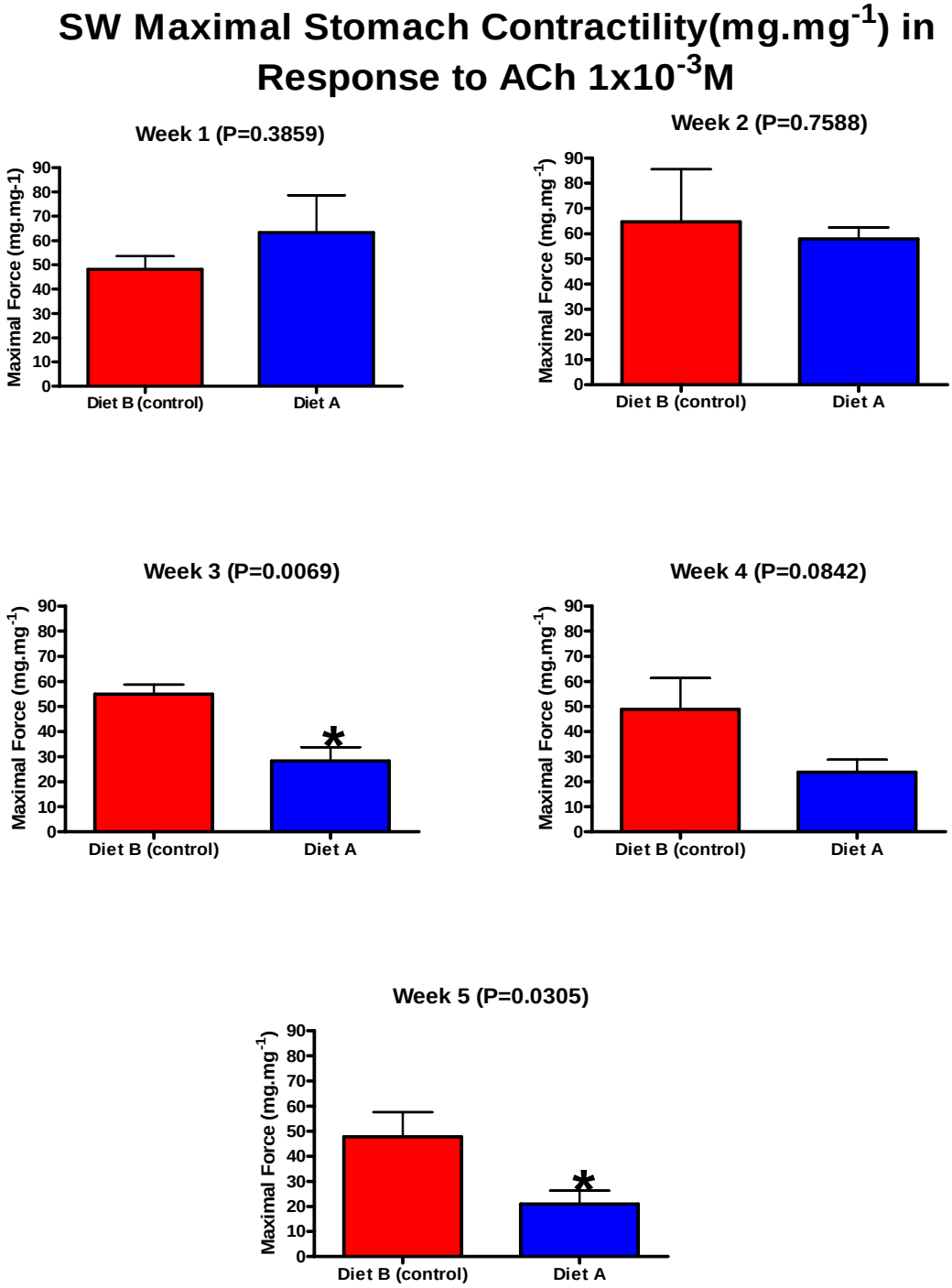

Figure 2.17. Weekly comparison of cardiac stomach circular smooth muscle maximal contractility ($mg.mg^{-1}$) in response to ACh $1x10^{-3}M$ between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red) in SW Chinook smolt. Unpaired two-tailed Student's t-tests showed significant differences in weeks 3 (P=0.0069) and 5 (P=0.0842). Bars that are significantly different are marked by a ***.** Data are mean ± SEM. n=4, except in weeks 4 and 5 where n=8 for each diet/week combination.

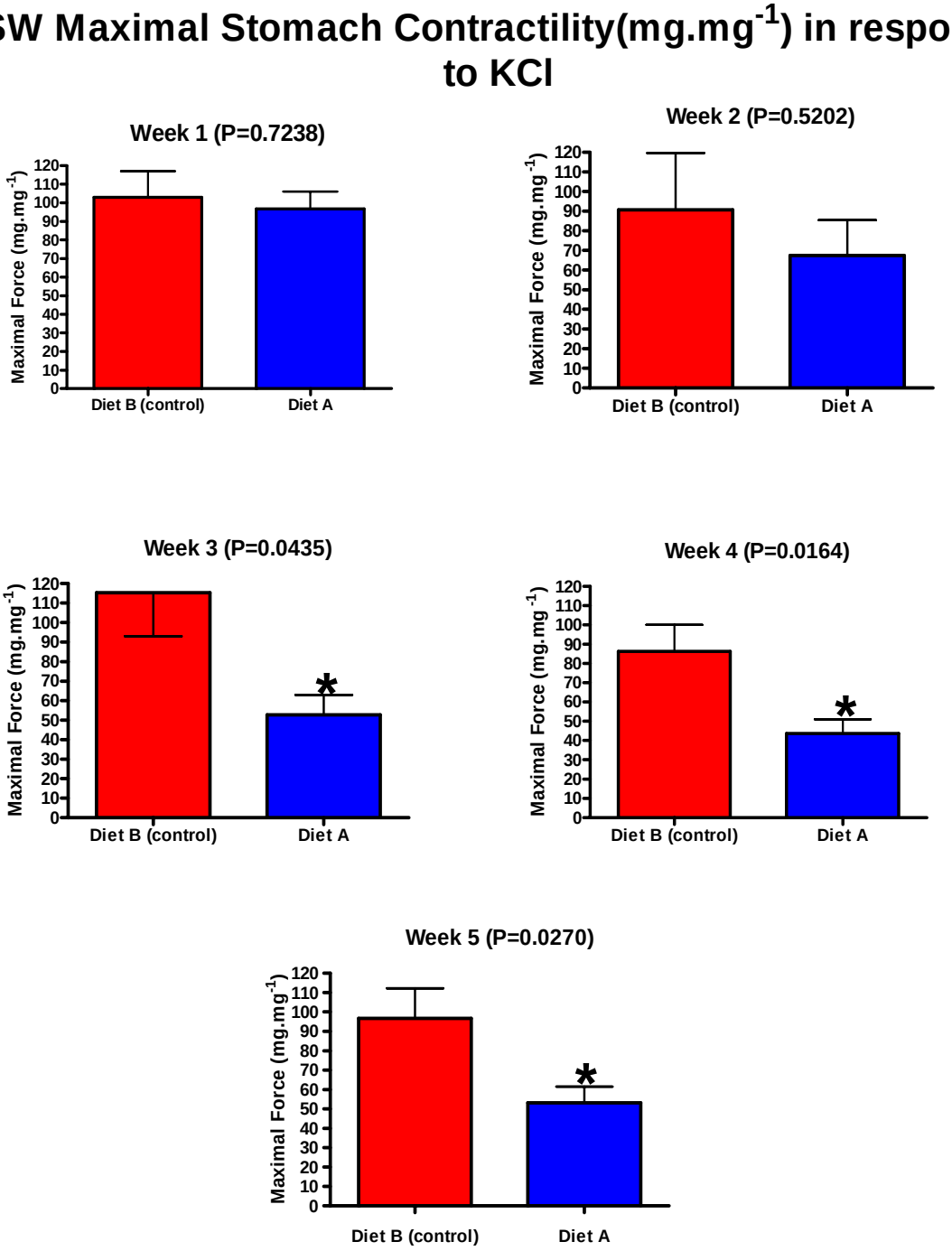

Figure 2.18. Weekly comparison of cardiac stomach circular smooth muscle maximal contractility (mg.mg^{-1}) in response to KCl between fish fed diet A (low cohesion, blue) and diet B (high cohesion, red) in SW Chinook smolt. Unpaired two-tailed Student's t-tests showed significant differences in weeks 3 (P=0.0435), 4 (P=0.0164) and 5 (P=0.0270). Bars that are significantly different are marked by a ***.** Data are mean ± SEM. n=4, except in weeks 4 and 5 where n=8 for each diet/week combination.

2.4.3 Gastric Evacuation

Fish fed diet B (control) in SW showed a linear trend of gastric evacuation (Figure 2.19). Fish fed diet A in SW showed a reverse exponential pattern of evacuation (Figure 2.19). The amount of digesta present in the stomach was found to be significantly reduced relative to controls after 4 hours when compared by an unpaired two-tailed student t-test (P=0.0075) (Figure 2.20). The amount of digesta present in the stomach was found to be no different relative to controls after 12 hours when compared by a unpaired two-tailed Student t-test (P=0.0906) (Figure 2.20). The amount of digesta present in the stomach was found to be significantly elevated relative to controls after 24 hours when compared by a unpaired two-tailed Student t-test (P=0.0077) (Figure 2.20).

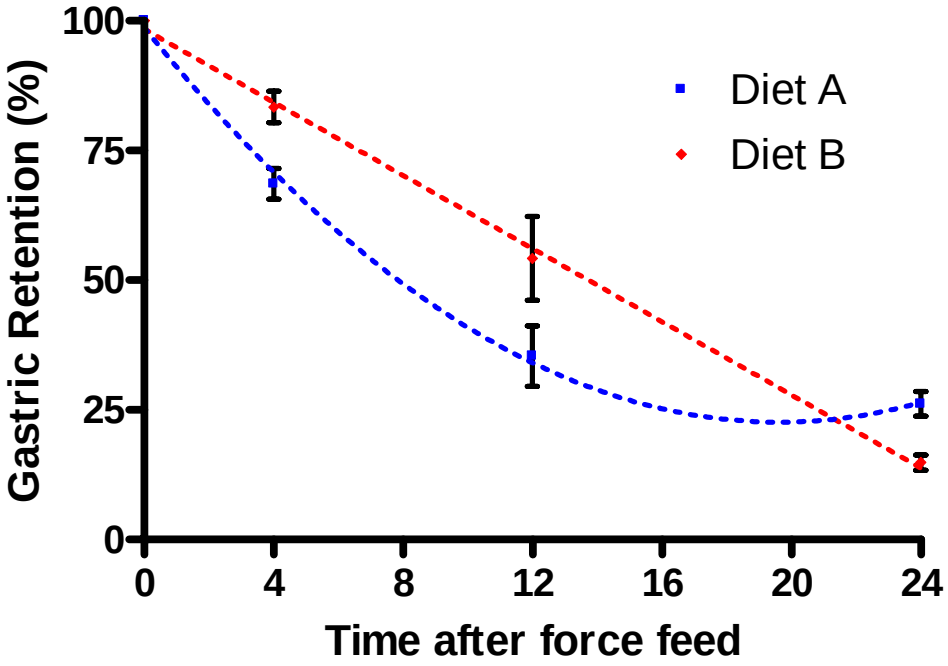

Figure 2.19. Pattern of gastric evacuation in SW acclimated Chinook smolt fed either diet A (low cohesion, blue) or diet B (high cohesion, red) over a 24 hour period. Diet B (control) was best described by a negative linear relationship (R^2=0.99, equation: y= -3.534x + 98.348) between gastric retention versus time. Diet A was best described by a negative exponential relationship R^2=0.88, equation: y= $0.1855x^2$ – 7.3713x + 96.376). Data are mean ± SEM. n=8 for each diet/week combination.

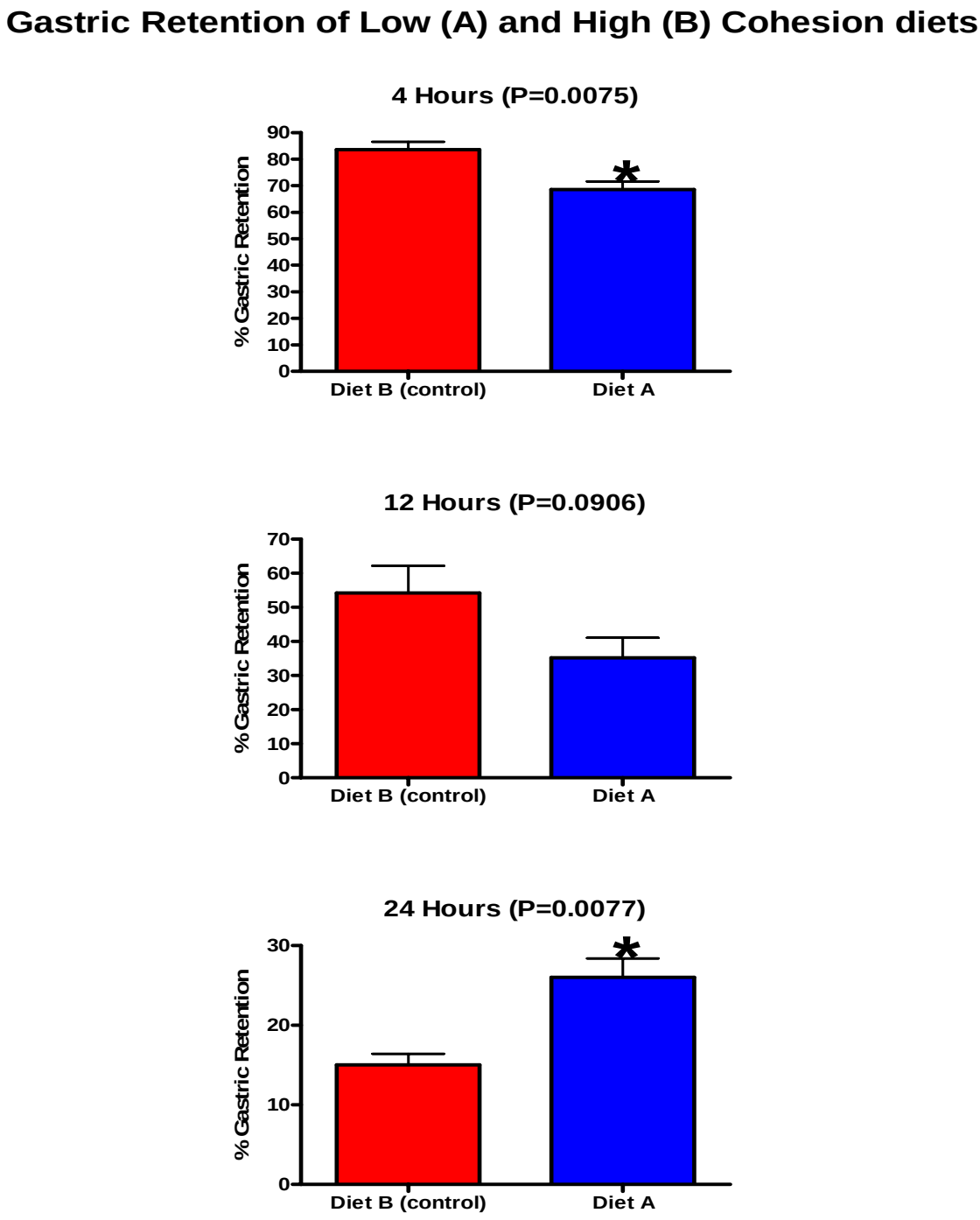

Figure 2.20. Gastric retention in the stomachs of fish fed either diet A (low cohesion, blue) or diet B (high cohesion, red) over a 24 hour period. Unpaired two-tailed Student's t-tests showed significantly lower diet A gastric retention after 4 hours (P=0.0075) relative to diet B. Although statistically non-significant, the same pattern was observed after 12 (P=0.0906). After 24 hours, diet A was significantly elevated relative to diet B (P=0.0077). n=8 for each diet/week combination.

2.4.4 Histology

Microscopic examination and analysis of the H & E stained cardiac stomach slides of fish fed either diet in FW showed no significant differences in overall morphology, therefore statistical analysis was not carried out on these data. Figure 2.21 is an image taken from a slide showing stomach tissue of a fish fed diet B for 6-weeks in FW. Figure 2.22 shows an image taken from a slide of stomach tissue of a fish fed diet A for 6-weeks in FW. These two sections show no major differences to other weeks and represent the morphology of a healthy stomach, based on measurements made by eye on a light microscope with a scale bar. Similarly, the SW diet B treatment (Figure 2.23) did not show any major differences in measurements when compared to the FW treatments. However, the SW diet A treatment (Figures 2.24-2.28) showed a decreasing mean smooth muscle width and an increasing inter-rugal fold distance over the 5-week period. Figure 2.24 shows a representative 'rugal' fold in a GDAS +ve fish fed diet A for 5-weeks. By week five, stomach distension in some fish was so severe that the rugal folds were almost indistinguishable from gastric pits. Figures 2.25-2.28 show representative section images from the 4 weeks prior to the final week of the trial, descending from week 4. Unpaired two-tailed Student's t-tests showed that smooth muscle width was significantly reduced ($P<0.0001$) (Figure 2.29A) in fish fed diet A compared with fish fed diet B for 5-weeks, while inter-rugal fold distance increased significantly ($P<0.0001$) (Figure 2.29B) over the same period.

Figure 2.21. H & E stained transverse section of FW Chinook salmon smolt cardiac stomach, fed on diet B for 5 weeks. Measurement 1 is the distance between the centre of a rugal fold and the centre of the fold immediately adjacent to it. Measurement 2 is the circular thickness of the circular smooth muscle layer. Magnification is 5X.

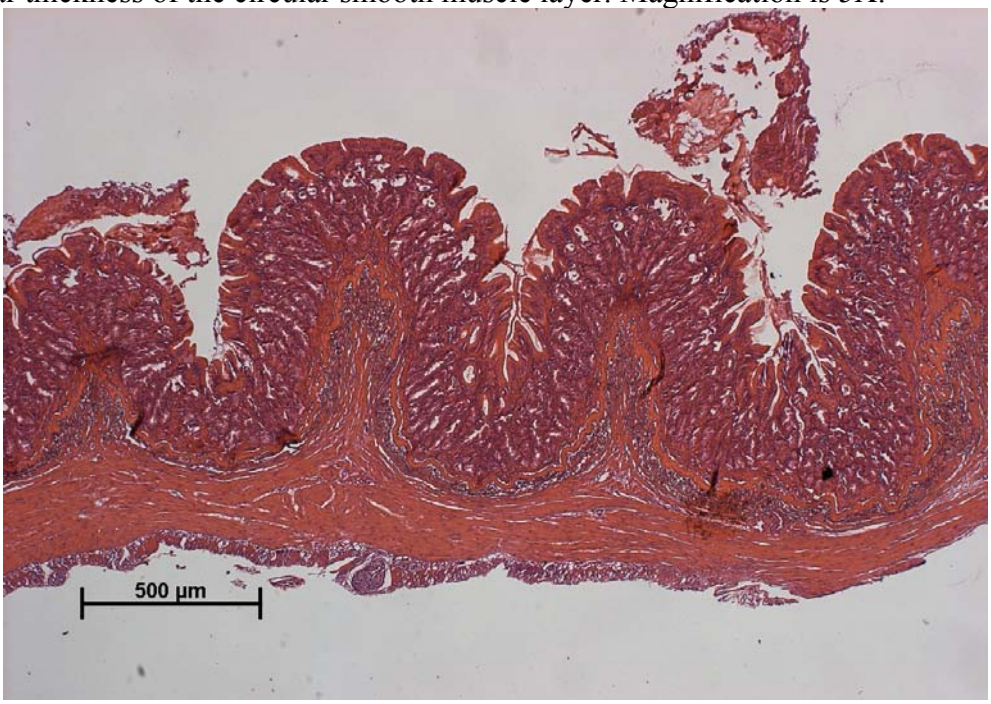

Figure 2.22. H & E stained transverse section of FW Chinook salmon smolt cardiac stomach, fed on diet A for 5 weeks. Magnification is 5X.

Figure 2.23. H & E stained transverse section of SW Chinook salmon smolt cardiac stomach, fed on diet B for 5 weeks. Magnification is 5X.

Figure 2.24. H & E stained transverse section of SW Chinook salmon smolt cardiac stomach, fed on diet A for 5 weeks. Magnification is 5X.

Figure 2.25. H & E stained transverse section of SW Chinook salmon smolt cardiac stomach, fed on diet A for 4 weeks. Magnification is 5X.

Figure 2.26. H & E stained transverse section of SW Chinook salmon smolt cardiac stomach, fed on diet A for 3 weeks. Magnification is 5X.

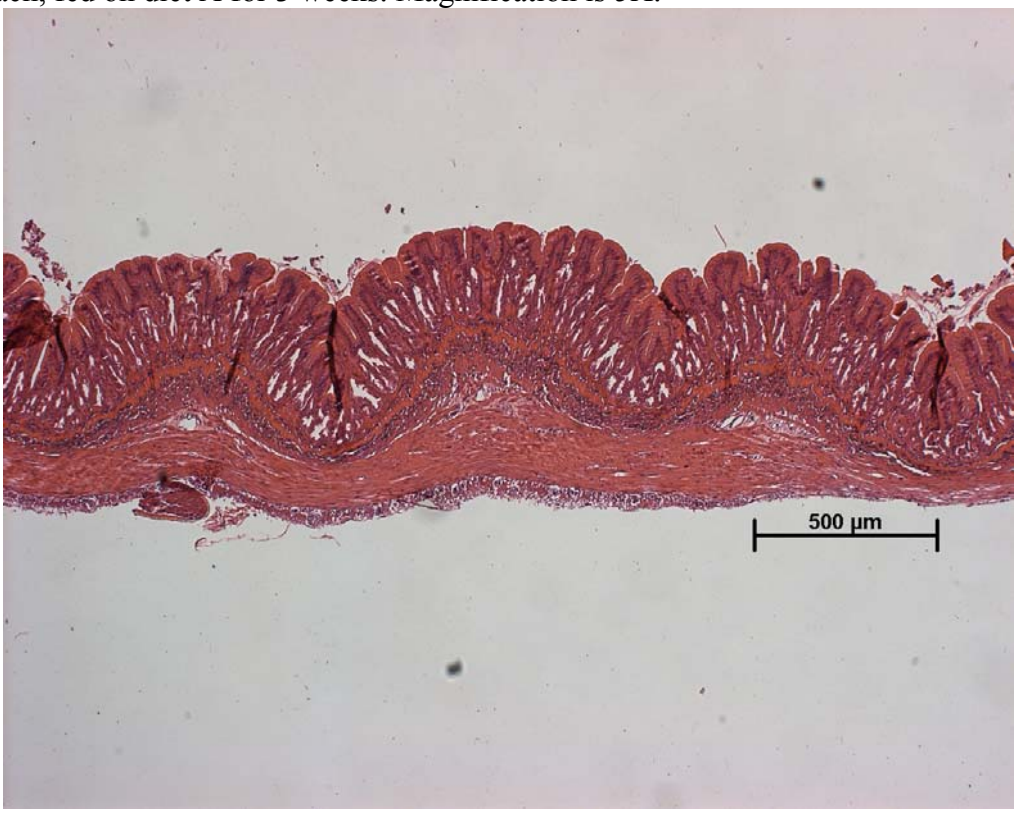

Figure 2.27. H & E stained transverse section of SW Chinook salmon smolt cardiac stomach, fed on diet A for 2 weeks. Magnification is 5X.

Figure 2.28. H & E stained transverse section of SW Chinook salmon smolt cardiac stomach, fed on diet A for 1 week. Magnification is 5X.

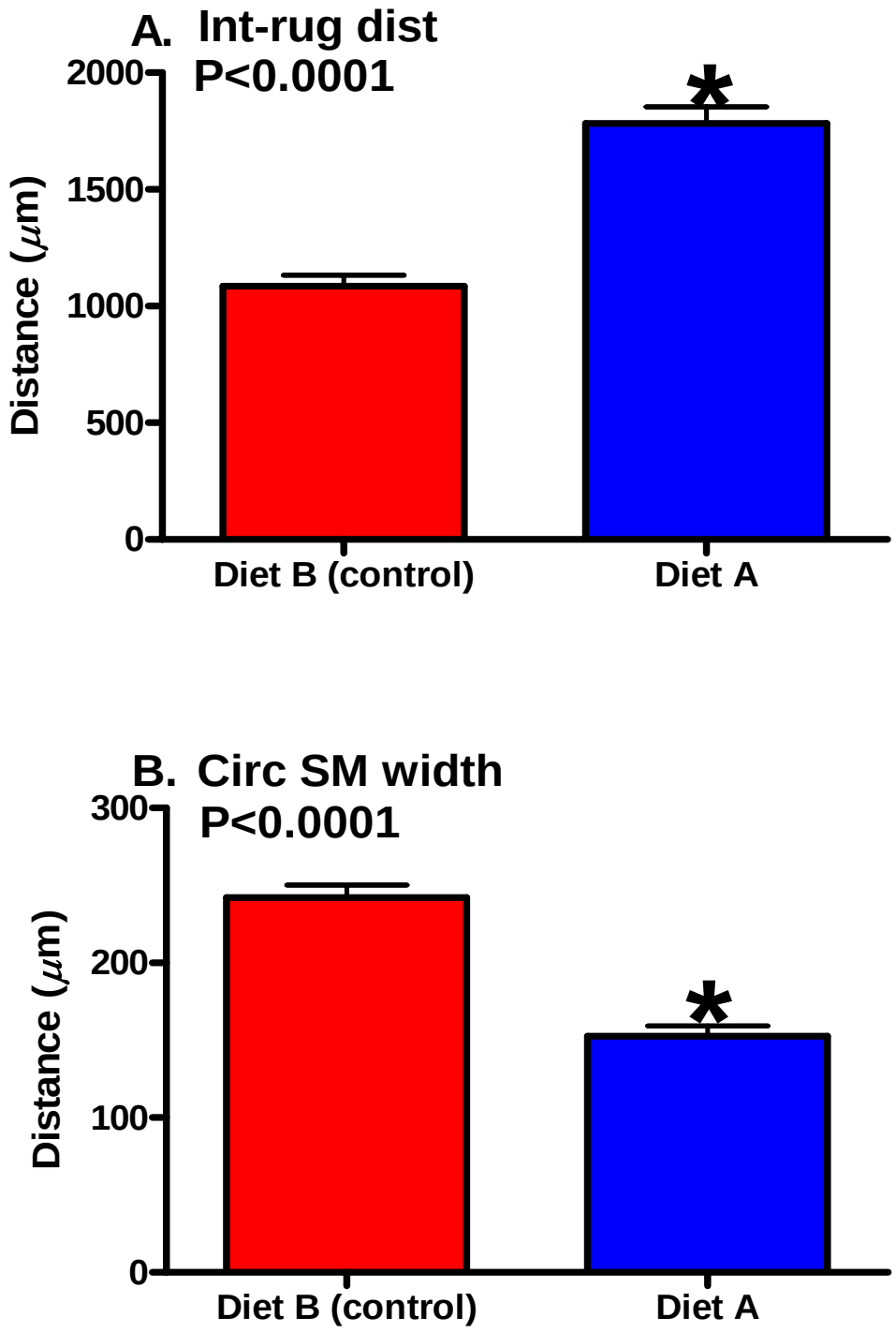

Figure 2.29. Summary of microscopic measurements taken from fish fed either diet A (blue) or diet B (red) for 5 weeks. All fish in the diet A treatment were GDAS +ve. A. shows the mean ± SEM inter-rugal fold distance. B shows the mean ± SEM circular smooth muscle width. * signifies significant differences between means, determined by two tailed unpaired Student's t-tests. n=3 for each diet.

2.5 Discussion

2.5.1 Feed Cohesion, GDAS Epidemiology and Physiology

My findings support the hypothesis that gastric dilation air sacculitis (GDAS) is caused by a lack of feed pellet cohesiveness and osmoregulatory stress, as was suggested by NZKS (pers. com.). In the trials (FW and SW) conducted in this study, diets of differing retention were fed; diet A (low cohesion) and diet B (high cohesion). These diets were commercially manufactured diets that had similar particle size (5-10μm) but different pellet cohesion properties. GDAS was induced only in SW smolt fed diet A (low pellet cohesion). This trend was not observed in FW smolt fed either diet nor in SW smolt fed diet B. This strongly implicates both low cohesion pellets and osmotic environment in GDAS development.

Commercially manufactured feed pellets vary in manufacture method, composition and physical properties. The three most important factors affecting the physical properties of a feed pellet are (1) composition, (2) particle size and (3) cohesion (Halver, 1989). These variables may have a profound effect on feed assimilation, growth, reproductive success and a whole host of other physiological systems (Halver, 1989). The GI system is no exception, with feed properties having far reaching consequences for function and health (Halver, 1989; Jobling, 1987). Inappropriately manufactured feed may cause gastrointestinal dysfunction (Jobling, 1987). Nutritional composition is vital in order to maximize tissue assimilation efficiency and prevent nutritional deficiencies (Halver, 1989). Care must be taken not to exceed the necessary amounts of those nutrients in order to maximise the profit margin of feed manufacturers and minimise the price of sale to aquaculture operations (Halver, 1989). Particle size of pelleted feed has been shown to

reduce the rate of digestibility between 5-10% compared to natural diets in teleosts (Jobling, 1986). Furthermore, diets composed of small particle size have been shown to overload the absorptive capacity of the intestine (Jobling, 1986). Jobling, (1987) has shown that finely ground high nutrient particles can effect what appears to have been an intestinal brake response. The rate of physical breakdown of this fine particulate matter is related to pellet cohesion in the stomach, and it appears that diets of low cohesion are easily disaggregated in the stomach (NZKS pers.com.). Jobling (1987) showed a reverse exponential pattern of gastric evacuation for diets with low nutrient small particle size, and presumably low cohesion. More solid food items were shown to conform to a linear pattern of gastric evacuation. This study has shown that diets with different cohesion are evacuated from the gut at different rates and in different patterns in healthy fish. Diet A fish showed a reverse exponential pattern of evacuation while diet B fish showed a linear pattern of gastric evacuation. Post-prandial serial slaughter of diet A fed fish showed that the feed pellets remain relatively intact in the stomach for quite some time after ingestion (NZKS pers. com; current study) and are thought to break down more gradually in the stomach, thus resulting in a low-medium nutritive chyme combining with ingested water and gastric secretions. Furthermore, the breakdown of all pelleted feeds in the fish stomach is thought to be slightly accelerated in SW due to the increased volume of water ingested, thus wetting the pellet. Consequently, it is thought that low cohesion pellets combined with SW mixing and physical breakdown in the stomach produces a high-nutrient chyme which moves quickly into the intestine. The drastic slowing of gastric evacuation in diet A fish after the initial rapid evacuation (reverse exponential) is thought

to be the result of nutritive feedback to the upper GI tract which effects a highly active intestinal brake, stronger than the response generated by diet B.

Nutritive feedback control from the intestine to the pyloric region of the stomach is well established in many vertebrate groups including fish (Olsson and Holmgren, 2001). Hence, in fish fed a low cohesion diet, the rate of passage of the food from the stomach to the intestine may be initially accelerated in diets of low cohesion, as chyme will form in the stomach and pass into the proximal intestine. Upon arrival, further enzymatic degradation occurs, and the processes of nutrient (Vieira and Baldisserotto, 2001) and water absorption (Rawdon and Cornish, 1973; Viellette, 2004) begin. Once this chyme reaches the proximal intestine, the intestinal brake is activated via nutritive chemoreception (Jobling, 1987; Olsson and Holmgren, 2001). This has been demonstrated in mammals (Vu *et al.* 2000) and fish (Olsson and Holmgren, 2001) and as explained in the introduction, the intestinal brake in fish parallels the ileal brake in mammals (Olsson *et al*, 1999; Olsson and Holmgren, 2001). The data presented in the current study suggest an overactive intestinal brake mechanism may be at work in the pathological development of GDAS.

With the intestinal brake mechanism activated, rate of high-nutrient chyme and H_2O transport into the intestine is drastically slowed (dos Santos and Jobling, 1988; Olsson *et al*. 1999). This is done so the chyme arrival rate does not exceed the absorptive capacity of the pyloric caecae and proximal intestine (Olsson *et al*. 1999). An overactive intestinal brake is predicted to slow this chyme and H_2O below normal levels. This creates a problem, as SW teleosts must constantly take up ingested water in order to maintain osmoregulatory homeostasis (Rawdon and Cornish, 1973). A maximally

stimulated pyloric sphincter tonus may prevent monovalent ion-associated water uptake, due to the prevention of ingested fluid moving into the intestine. Evidence for this can be seen in the serum osmolality data. Diet A fish have higher serum osmolality than diet B fish, representing dehydration in those fish. This may suggest that there is less osmotically free water available for absorption in the caecae and intestine. This trend is also present in the osmoregulation potential data of the isolated pyloric caecae preparations. The impaired osmoregulatory homeostasis in these fish may reflect a relative downregulation of intestinal enterocyte cells or impaired cell function. This may occur as a response to reduced fluid flow into the intestine. This could be supported by measuring Na^+-K^+-ATPase activity in intestinal pyloric cecae epithelial tissue extracts (Viellette, 2004) and extrapolating enterocyte activity, or by using histological methods.

With the constant need to drink, accelerated by the increased serum osmolality and the increased tonus of contraction of the pyloric sphincter suggested in the intestinal brake hypothesis (Chapter 1), massive pressures are most likely created in the stomach by continual drinking into a 'closed space' (the stomach). This almost certainly results in the massive distension seen in GDAS +ve fish. These pressures could force stomach contents into the swimbladder via the pneumatic duct, resulting in the viscous fluid, oil and diverse bacterial fauna reported in chronically affected individuals (Lumnsden *et al*. 2002).

Contractility of circular smooth muscle from cardiac stomach in SW adapting smolt fed diet A was shown to be progressively lost from week 3 of the SW trial. This was apparent in all three treatments (ACh $1x10^{-4}$ M, ACh $1x10^{-3}$ M and KCl). A concomitant increase in maximal pyloric sphincter circular smooth muscle contractility

was observed in SW smolt from the 3rd week on diet A. The loss of contractility seen in cardiac stomach circular smooth muscle is consistent with Laplace's Law (P= 2T/r), suggesting that an increasing radius will require an increase in muscle mass to achieve the same degree of contraction. Consequently, it was predicted that if stomach muscle mass does not increase then muscle tone will be lost. This is shown to be true in SW smolt fed diets of low cohesiveness (myography and histology). A concurrent loss of control may also be occurring, as the ACh sensitivity appears to have also decreased, since ACh treatments provide insight into cholinergic receptor initiated activity. A decrease in contractility is suggestive of either a downregulation in muscarinic receptors or impaired ligand – receptor function (Sanders and Ozaki, 1994). The main mammalian muscarinic receptor subtypes in the digestive tract are M_2 and M_3 (Ehlert *et al.* 1997), which are coupled to G-proteins. These receptors effect different intracellular processes. M_2 acts predominantly via inhibition of the enzyme adenylate cyclase and hence decreases the amount of cAMP in the cell, while M_3 activates phospholipase C, which hydrolyses phosphatidyl inositol biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (Ehlert *et al.* 1997). In teleosts, muscarinic receptor distribution is not as clear cut. Recent data published by Olsson and Holmgren (2001) suggest that the M_3 is the main receptor type responsible for smooth muscle contraction, with M_2 most likely playing a lesser role. Receptor abundance in GDAS positive intestinal tissues may reveal conclusive results on receptor distribution post-GDAS development. The data presented here may suggest either a downregulation in receptors in tissues or decreased sensitivity to ACh, or simply an inability of the smooth muscle to contract in response to ACh treatment.

An alternate explanation for the reduced responsiveness of GDAS +ve cardiac stomach preparations to ACh could simply be impaired muscle function. A decreased responsiveness may represent a fundamental loss of muscle control or processes that control contraction. The loss of function demonstrated by KCl treatment could be indicative of impaired localised ACh release or responsiveness of smooth muscle populations. These data together certainly suggest fundamental changes in responsiveness and normal function.

Understanding the pharmacology of GI smooth muscle is difficult, as a multitude of naturally occurring and synthetic substances affect the electrical and contractile behaviours of these muscles (Sanders and Ozaki, 1994). Another complicating factor in understanding the excitation-contraction coupling in GI smooth muscle is that the basic physiology of these muscles varies extensively in different regions of the GI tract (Sanders and Ozaki, 1994). Therefore, it was difficult to interpret the smooth muscle activity observed in the current study. However, correlates were drawn based on the best evidence available at the time of going to press. The *in vitro* increase in pyloric sphincter circular smooth contractility in SW smolt fed diet A is consistent with the intestinal brake hypothesis (Chapter 1). The greater force of contraction is interpreted as the result of changes in stimuli acting upon the pyloric sphincter, in order to maintain closure between the two compartments (stomach and intestine) when stomach intraluminal pressure is very high. Therefore, these data may suggest an increase in the muscularity of the pyloric sphincter. This is probably a result of prolonged elevated smooth muscle tonus in the pyloric sphincter which increases muscularity, or possibly due to an increase in muscarinic receptors in the pyloric sphincter region (Vu *et al.* 2000; Maes *et al.* 1998).

Earlier work on the syndrome, examining chilling stress, (Róvric, 2000) and histamine (Wantanabe, 1987; Fairgrieve, 1994) as causative factors of 'bloat' in fish may have been inadvertently inducing GDAS or pre-GDAS symptoms by feeding a low-cohesion feed in their experimental trials. This is even more likely to have occurred if the feed pellets were modified or re-pelleted (i.e. moisture content increased/decrease by pharmacological agent addition in solution and/or the physical degradation of the pellets in order to incorporate agents into subsequently reformed pellets.). Alternatively, those trials may have induced other GI syndromes. The syndrome GDAS, and its associated physiological dysfunction in commercial SW operations is most likely solvable by feeding high cohesion pelleted feeds with particle sizes as large as commercial manufacturing operations will allow. This should be strictly monitored by industry in order to prevent GDAS incidence. Potential reversibility of the syndrome will be discussed in the general discussion (Chapter 4).

In summary, this study has shown GDAS to occur only in SW smolt fed a diet with low physical cohesion properties. This is hypothesised to be the result of intestinal brake dysfunction. Physical properties (cohesion and particle size) were shown to affect gastric emptying in SW fish. In addition, GDAS smolt GI circular smooth muscle tissues and osmoregulatory ability were shown to be dysfunctional. Furthermore, high retention pelleted feed is highly unlikely to result in GDAS in SW aquaculture operations.

CHAPTER 3

Control of the Intestinal Brake and the Effects of CCK8, Gastrin-1, GLP-1 and 5-HT on Gastrointestinal Smooth Muscle in Chinook Salmon (*Oncorhynchus tshawytscha*)

3.1 Abstract

The intestinal brake (IB) in fish is known to be under humoral and neural control. Potential humoral factors controlling the IB in Chinook salmon were characterised in terms of their effect on frequency and amplitude of gastrointestinal smooth muscle spontaneous contractions *in vitro*. The neurotransmitter 5-HT was also investigated to contrast this data. Concentration-response curves of gut contractility were produced for Cholecystokinin-8 (CCK8), Gastrin-1, Glucagon-like-peptide-1 (GLP-1) and serotonin (5-HT) for cardiac stomach (CS), pyloric stomach (PS), pyloric sphincter (PSp) and intestinal (Int) circular smooth muscle rings. Mean peak force (PF) ± SEM and rate of spontaneous spike frequency (SPM) were also measured for each hormone/neurotransmitter-tissue combination. Log10 EC_{50} (± CI) and PF (± SEM) values calculated for CCK8 (n=7) were; CS log10 EC_{50} -8.15 ± 0.90, PF 46.8 ± 13.2; PS log10 EC_{50} -7.88 ± 0.48, PF 32.2 ± 9.6; PSp log10 EC_{50} -8.98 ± 0.68, PF 155.2 ± 59.5; Int log10 EC_{50} -8.93 ± 0.64, PF 69.0 ± 20.0. With the exception of the pyloric sphincter, log10 EC_{50} values for gastrin-1 (n=7) were lower, demonstrating higher sensitivities, while PF values were similar; CS log10 EC_{50} -12.45 ± 0.66, PF 36.3 ± 12.6; PS log10 EC_{50} -12.55 ± 0.63, PF 42.5 ± 1.0; PSp log10 EC_{50} -9.35 ± 0.78, PF 162.0 ± 56.3; Int log10 EC_{50} -12.69 ± 1.12, PF 56.0 ± 10.9.

Log EC_{50} and PF values calculated for 5-HT (n=6) were; CS log10 EC_{50} -4.78 ± 1.05, PF 7.5 ± 2.1 and PSp log 10 EC_{50} -6.18 ± 1.14, PF 3.3 ± 1.2. Thus the amplitude of the force generated in response to 5-HT was considerably less, while the concentration required to elicit half the maximal response was much greater. Linear regression of SPM showed a significantly negative trend for CS in response to increased concentrations of CCK8 (P=0.0012) and gastrin-1 (P=0.0423), while 5-HT produced a significantly positive relationship (P=0.0094). PSp tissue showed significant positive relationships in response to increased concentrations of both CCK8 (P=0.0132) and gastrin-1 (P=0.0004). GLP-1 (n=4) produced no response in any of the tissues examined between the concentration range $1x10^{-13}$ M – $1x10^{-6}$ M. Blood borne factor or factors potentially capable of producing an intestinal brake response in the serum of fish diagnosed with gastric dilation air sacculitis (GDAS) were found to be elevated above controls (no discernable GDAS). Myography of circular smooth muscle rings revealed that sequential additions of 1000- and 100-fold serum dilutions produced a significantly elevated tonus of the cardiac stomach (1000-fold, n=6, P=0.0016; 100-fold, n=6, P=0.0423) and pyloric sphincter (1000-fold n=6; P=0.0003; 100-fold, n=6, P=0.0169).

3.2 Introduction

The intestinal brake (IB) in fish is known to be under humoral control (Olsson and Holmgren, 2001). The IB results in an increase in the force and a decrease in the rate of peristaltic contractions in the stomach (Olsson and Holmgren, 2001). It also contracts the pyloric sphincter with sufficient force to prevent peristaltic wave generated intraluminal pressure opening. The net result of these actions is to slow the rate of food evacuation from the stomach (Olsson *et al*, 1999; Olsson and Holmgren, 2001). This is done to prevent the absorptive capacity of the proximal intestine being exceeded (McCormick, 2001). The syndrome GDAS in Chinook salmon is hypothesised to be the result of an IB dysfunction (intestinal brake hypothesis; Chapter 1 & 2). Therefore, it was hypothesised that (1) the blood of GDAS +ve fish would contain higher concentrations of factors capable of producing an IB response than GDAS -ve fish and (2) that known gastrointestinal (GI) peptides would exert similar effects to those blood borne factors. Hence, GDAS -ve and +ve serum was investigated for its potential effects on cardiac stomach and pyloric sphincter smooth muscle rings *in vitro*. In addition, humoral factors potentially involved in the control of the IB in Chinook salmon were investigated in terms of their effect on frequency and amplitude of GI circular smooth muscle spontaneous contractions *in vitro*. The *in vitro* approach was determined to be informative due to the ability to exclude extrinsic neural activity as an explanation for the observed responses. Tissues used were cardiac stomach (CS), pyloric stomach (PS), pyloric sphincter (PSp) and intestinal (Int) circular smooth muscle rings. The humoral factors used were selected for investigation based on published data on the control of gastric evacuation in vertebrates (Holmgren *et al.* 1982; Matty, 1985; Olsson *et al*, 1999;

McCormick, 2001; Olsson and Holmgren, 2001; Shirra and Göke, 2004). These peptides were determined to be the most likely effector molecules of the IB in Chinook salmon. IB dysfunction may be correlated with increased plasma concentrations of one or more of these factors. It was hoped that similar patterns of activity would be induced by pharmacological application of one or more of the humoral factors and the GDAS +ve serum. To the knowledge of the author, this is the first time these factors have been applied to the Chinook GI tract in a pharmacological study and will therefore be informative in closing the gaps in the understanding of salmonid GI control.

3.2.2 Hormonal Control of Gastric Evacuation

Liddle (1986) has shown that in humans the predominating mechanism for reducing gastric evacuation rate after the arrival of food in the intestine is the ileal brake, which is mediated by the release of duodenal cholecystokinin (CCK) and gastric gastrin. Similarly, Olsson *et al.* (1999) have shown an important role for CCK8 in the control of gastric evacuation in fish. However, CCK and gastrin do not appear to be the only molecules responsible for the control of gastric evacuation, with several other hormones and neurotransmitters implicated (Olsson and Holmgren, 2001). These include acetylcholine (ACh) noradrenaline (NA), tachykinins, serotonin (5-HT), vasoactive intestinal poplypeptide (VIP), motilin and galanin (Olsson and Holmgren, 2001). In addition, Orihata and Sarna (1994) have shown that nitric oxide synthase delays gastric evacuation, mainly due to the lack of co-ordination between different parts of the stomach and an increased tonus of the pyloric region in mammals. Glucagon-like-peptide-1 (GLP-1) (Chelikani *et al*. 2005) has also been shown to be important in gastric

evacuation in mammals. Not all the humoral factors/neurotransmitters listed here were investigated due to time and budget constraints, but CCK8, gastrin-1, GLP-1 and 5-HT were investigated for their effects on GI smooth muscle activity in SW acclimated Chinook smolt. Current data on these peptides are reviewed below.

3.2.3 Gastrointestinal Activity of the GI tract in Response to CCK8, Gastrin-1, GLP-1 and 5-HT

The neuroendocrine GI peptides CCK and gastrin, originally identified in mammals, are characterised by a common amidated N-terminal tetrapeptide sequence, Gly-Trp-Met-Asp-Phe•NH_2, which also constitutes the minimal structure necessary for biological activity of both (Johnsen, 1998). Hence, it is generally accepted that CCK and gastrin share a common ancestor; however gastrins and CCKs have been shown to exert very different actions, as their names imply (Johnsen, 1998). The CCK/gastrin family appears to be represented in most, if not all chordates (Johnsen, 1998). The active site is present in such primitive groups as coelenterates, suggesting strong conservation over evolutionary time (Rehfeld and Hensen, 1984). Evidence for the CCK/gastrin active sequence in invertebrates is lacking and it appears that CCK and gastrin probably arose as distinct GI peptides in early vertebrate history (Johnsen, 1998). While CCK is conserved in most vertebrates, gastrin appears to have differentiated significantly during the evolution of mammals (Johnsen, 1998). Both peptides are known to play crucial roles in the control of the GI tract of vertebrates (Ganong, 1977). Control mechanisms and current knowledge of these peptides are discussed below. Glucagon-like-peptide-1 (GLP-1), one of the proglucagon derived peptides, has important physiological functions in vertebrates (Ruppin and Domschke, 1980). In mammals, it has been shown to stimulate

glucose dependent insulin secretion, inhibition of glucagon secretion and antropyloroduodenal motility (Yeung *et al*. 2002) and appetite (Chelikani, 2005), However, its role in appetite is unclear and it may cause stimulation or inhibition Chelikani, 2005). Its role in non-mammalian vertebrates is virtually uncharacterised. Current data on GLP-1 are presented below. 5-Hyroxytryptamine (5-HT) has also been implicated in the control of the vertebrate GI tract (Olsson and Holmgren, 2001). Current knowledge of its involvement in GI activity is also presented below.

3.2.3.1 Gastrin

The gastrin family of peptides is a group of GI polypeptides that are found in higher vertebrates (Matty, 1985; Kurokawa *et al*. 2003). There is a sulphated tyrosine near the C-terminal in gastrin (Ganong, 1977). It was the first GI hormone for which the chemical structure was determined (Gregory *et al.* 1969) and is produced by G-cells in the antral portion of the gastric mucosa in the stomach of mammals (Matty, 1985) and the intestinal mucosa in fish (Olsson and Holmgren. 2001).

Gastrin is primarily responsible for stimulating GI motor activity (peristalsis) (Ganong, 1977) and secretion of gastric acid and pepsin into the stomach (Gregory *et al.* 1969). It also has a trophic action in specifically stimulating growth of the stomach and intestinal mucosa (Ruppin and Domschke, 1980). Gastrin activity has been shown to be present in numerous vertebrate groups, with multiple forms of the peptide isolated (Matty, 1985). The most well represented group in the literature are the mammals with fish receiving relatively little attention (Matty, 1985). Gastrin genes have been isolated from two teleost species, the puffer and the flounder (Kurokawa *et al.* 2003). Kurokawa

et al. (2003) showed that puffer gastrin open reading frame cDNA contained 678 bp and that flounder mRNA contained 526 bp

Gastrin has activity in most of the GI tract, but most of this appears to be pharmacological (Matty, 1985). Isolated strips of human, cat, guinea pig and hamster fundus or antrum are contracted by natural or synthetic 'little gastrin' (Gastrin-17; EC_{50} $4.8x10^{-12}$ M), tetragastrin (TG; EC_{50} $5x10^{-12}$M), and pentagastrin (PG; EC_{50} $1x10^{-12}$ M) (Ruppin and Domschke, 1980). Mammalian gastrin pharmacology has revealed that isolated antral preparations increase frequency and amplitude of phasic motor activity, while fundic preparations mainly show an elevation of the muscular tonus (Ruppin and Domschke, 1980). In rainbow trout, Holmgren *et al.* (1982) have shown that gastrin-1 contracts the stomach. The pattern of mammalian gastric motor activity *in vitro* is similar to that *in vivo* (Ruppin and Domschke, 1980), and gastric smooth muscle is a robust tissue (pers. observation). Gastrin and its analogs exert their effects partly *via* post-ganglionic cholinergic fibres, stimulating the release of ACh, and partly via direct interaction with muscle cell receptors (Matty, 1985). Some work investigating gastrin activity has suggested that it may delay gastric evacuation of both liquid and solid meals in mammals (Ruppin and Domschke, 1980). In addition, high concentrations of gastrin have been shown to contract the pylorus in humans (Ruppin and Domschke, 1980). However, this effect is probably analogous to the effect on antral stomach activity, as physiological concentrations of the hormone eliciting one-half maximal gastric acid secretion (D_{50}) have been shown not to affect stomach evacuation of liquids in mammals (Ruppin and Domschke, 1980).

When gastrin production is inadequate or excessive, GI dysfunction is known to occur (Ganong, 1977). Low gastrin production has been linked with gastroesophageal reflux while excessively high gastrin production has been linked with the disease achalasia (Ganong, 1977). Achalasia is a disease of the oesophagus, which results in food accumulation in the oesophagus because the gastroesophageal sphincter fails to relax (Ganong, 1977). In addition, normal peristaltic activity in the oesophagus is impaired and the oesophagus becomes massively dilated (Ganong, 1977). Microscopic examination of the wall of the dilated oesophagus reveals a deficient myenteric plexus (Ganong, 1977). This has parallels with the syndrome under investigation in the current study (GDAS). Analogous to the gastroesophangeal sphincter dysfunction in achalasia in human patients, the pyloric sphincter in GDAS affected fish is hypothesised, in the current study, to become strongly contracted when peristaltic waves terminate in the pylorus, preventing inter-compartmental digesta transport. The net outcome of this action is thought to result in massive dilation of the stomach, due to impeded digesta movement and continual drinking. Myenteric plexus depletion has been shown in GDAS affected Chinook salmon dilated stomach sections (Lumsden *et al.* 2003).

In order to further clarify the role of gastrin in fish (Chinook salmon) and to investigate it as a potential humoral factor involved in the intestinal brake hypothesis through a potential overproduction of the peptide, spontaneously contracting CS, PS, PSp and int circular smooth muscle strips were pharmacologically challenged by increasing concentrations of synthetic human gastrin-1.

3.2.3.2 Cholecystokinin (CCK)

Originally purified from the pig (Mutt, 1968), cholecystokinin (CCK) was thought to produce contractions of the gallbladder, whereas a separate hormone called pancreozymin (PZ) increased the secretion of pancreatic juice rich in enzymes in humans and other mammals (Ganong, 1977). It is now known that both responses are elicited by the same hormone, known as CCK-PZ or just CCK (Ganong, 1977). Since this discovery, several related peptides of the CCK family have been identified in several vertebrate groups, with multiple targets. The active part of the molecule, an octapeptide found at the N-terminal end is present in most forms of the peptide, with the last five of these amines identical to those in found at the N-terminal of gastrin. In addition, there is a sulphated tyrosine near the N-terminal in CCK just as there is in gastrin (Vigna, 1985). Removal of the $–SO_3H$ group changes the activity patterns of CCK (and gastrin) (Ganong, 1977).

After the discovery of gastrin and CCK in mammals, the opportunity to investigate the presence, and potentially study the actions, of these peptide hormones in phylogenetically ancient vertebrate groups presented itself. The identity of the biologically active part of both gastrin and CCK has led to the hypothesis that CCK and gastrin originate from a common ancestor (Johnsen, 1998). A CCK-related peptide known as cionin was identified in the protochordate, *Ciona intestinalis* and contains a N-terminus common to mammalian CCK and gastrin and has tyrosine in both position 6 and 7. It is a structural hybrid of the CCK/gastrin families (Jensen *et al*. 2001). Sequencing, biological assay and immunocytochemical studies indicate a CCK-like hormone present in cyclostomes and teleosts (Jensen, 2001) as well as in two cartilaginous fishes

Scyliorhinus stellaris (Cimini *et al.* 1985) and *Lamna cornubica* (Johnsen, 1996). This suggests that CCK evolved early in chordate evolution.

Jönsson *et al*. (1987) have shown gastrin/CCK-like immunoreactivity in endocrine cells and nerves of the GI tract of the Atlantic cod, *Gadus morhua*. Furthermore, in the cod isolated stomach strips contracted in response to the application of human sulphated gastrin-5 and -17. Later, three CCKs were isolated and sequenced in rainbow trout (Jensen, 2001). Later still, gastrin and multiple CCK genes were identified in the puffer and the flounder (Kurowara *et al.* 2003). Jensen *et al*. (2001) have identified and characterised the distribution of CCK related peptides and mRNAs in the rainbow trout. They identified three CCK-related molecules (CCK-N, CCK-L and CCK-T), with all forms identified determined to be fully sulphated. They found that CCK-N and CCK-L (both octapeptides) were present in equal amounts in the brain, while CCK-T was confined to distinct but different parts of the brain in lesser amounts. Three forms of CCK-L were determined to be present in the pyloric caecae (CCK-7, CCK8 and CCK-21: these contained 7, 8 and 21 residues respectively). It would follow that these forms, or some very similar form, will occur in other species in the genus *Oncorhynchus*.

CCK was initially discovered to cause gallbladder contraction in mammals. Since this time it has been shown to have analogous activity in other vertebrate groups, for example fishes (Olsson and Holmgren, 2001). CCK-related peptides have been shown to cause gallbladder contractions in bluefish, killifish, bowfish and rainbow trout (Einarsson *et al*. 1997). Another significant action of CCK in both mammals and fishes is the secretion of pancreatic proteases (Einarsson *et al*. 1997). Einarsson *et al*. (1997) have demonstrated the dose-dependent release of both trypsin and chymotrypsin from both the

pyloric caecae and pancreatic tissue of Atlantic salmon (*Salmo salar*) in response to intraperitoneal injection of purified porcine CCK. A similar physiological role has been reported for sharks, as CCK-like substances have been detected in pancreatic and intestinal tissues (Gelsleichter, 2004).

CCK has also been shown in humans, rats, guinea pigs and dogs to increase basic tension and amplitude of contractions of both fundic/cardiac and antral/pyloric preparations and to contract the pyloric sphincter in all groups *in vitro* and *in vivo* (Ganong, 1977; Ruppin and Domschke, 1980; Moran *et al*. 1994; Einarsson *et al*. 1997; Olsson and Holmgren, 2001). Similarly, CCK8 has been shown to contract rainbow trout circular and smooth muscle strips *in vitro* (Holmgren*, et al.* 1982*).* Chey *et al*. (1970) have demonstrated the inhibitory action of physiological concentrations of CCK on gastric evacuation in humans. Similarly, in the dog the inhibition of gastric evacuation has been suggested as a physiological function of endogenous CCK (Ruppin and Domschke, 1980). The delayed gastric evacuation observed is thought to be attributable to CCK-induced pyloric sphincter closure (Debas *et al*. 1975). Debas *et al*. (1975) found that in dogs, administration of CCK8 at a physiological concentration inhibited the evacuation of a 0.15M NaCl liquid meal. Similarly, in rainbow trout, CCK8 has been shown to slow gastric emptying *in vivo* (Olsson *et al*, 1999).

The principal stimulus for CCK secretion is the presence in the intestine of fatty acids containing more than 10 carbon atoms in mammals and 8 carbon atoms in fish (Hardy, 2002). Its secretion is also increased by the presence of amino acids in the intestine, particularly tryptophan and phenylalanine, and by Ca^{2+} and H^{+} (Ganong, 1977). In mammals the bile and pancreatic enzymes that enter the duodenum in response to

CCK further the digestion of protein and fat and the products of this digestion. In turn, this stimulates further CCK secretion (Ganong, 1977). Hence, a positive feedback operates in the control of this hormone (Ganong, 1977). The positive feedback is terminated when the products of digestion move to the lower portions of the GI tract (Ganong, 1977).

In order to evaluate the effects of CCK on the Chinook salmon GI tract and investigate it as a potential humoral factor involved in the intestinal brake hypothesis through a potential overproduction of the molecule, spontaneously contracting CS, PS PSp and Int circular smooth muscle strips were pharmacologically challenged by increasing concentrations of synthetic sulphated human CCK8.

3.2.3.3 Glucagon-Like-Peptide-1 (GLP-1)

GLP-1 is an intestinal peptide post-prandially released by L cells of the lower gut (upper- and lower-intestine; concentrated in the ileum and colon) in mammals (Schirra and Göke, 2004). It has been shown experimentally to control food intake (Chelikani *et al*. 2004), plasma glucagon levels and stimulate insulin secretion as well as all the motor mechanisms known to control gastric evacuation in mammals (Schirra and Göke, 2004). Hence, it is both an incretin (insulin secreting) hormone and an enterogasterone. In their recent review, Schirra and Göke (2004) outline how the synthetic molecule has been successfully shown by numerous workers to exert (1) a glucose dependent insulinotrophic effect at the pancreatic β-cells, (2) lower plasma glucagon by exerting an inhibitory effect against the α-cells, and (3) delay gastric evacuation by the relaxation of the fundus and inhibition of antral contractility in addition to the stimulation of both the

tonus and phasic motility of the Psp. The inhibitory effect on the upper GI functions is at least partly mediated by vagal-cholinergic inhibition and may involve interactions with vagal afferent pathways and/or circumventricular regions within the CNS. In humans (Brennan *et al*. 2005) and rats (Chelikani *et al*. 2005) GLP-1 is a candidate humoral mediator of the ileal brake, exerting inhibition of upper GI function, thus preventing malabsorption and post-prandial metabolic disturbance. GLP-1 has been shown to act upon cell surface receptors (Yeung *et al*. 2002). The GLP-1 receptor (GLP-1R) belongs to a large family of seven membrane-spanning G-protein-coupled receptors (GPCRs), which also includes receptors for secretin, calcitonin, growth hormone-releasing hormone (GHRH), glucose-dependent insulinotrophic polypeptide (GIP) and glucagon (Yeung *et al*. 2002). Many GPCRs activate adenylyl cyclase which mediates signal transduction (Yeung *et al*. 2002), while others mediate their actions through other enzymes, for example the previously mentioned (p67) muscarinic receptor (M3) activation of phospholipase C (Ehlert *et al.* 1997).

Brennan *et al*. (2005) investigated whether there was a synergistic relationship between between sulphated CCK8 and GLP-1 in their effects on appetite, energy intake and antropyloroduodenal motility in healthy men in their early twenties. At the physiological concentrations they evaluated, exogenously administered CCK8 and GLP-1 in combination displayed no synergy; they did not exceed the sum of the effects of CCK8 and GLP-1 administered alone.

Enç *et al*. (2001) showed that acarbose, a complex pseudo-oligosaccharide of microbial origin with an inhibitory ability to bind to α-glucosidases and pancreatic α-amylases reversibly, alters carbohydrate absorption and thus modifies gastric evacuation

of a meal. Their work showed that acarbose delays gastric evacuation by augmenting GLP-1, with CCK8 and peptide YY playing contributory roles. They concluded that when acarbose is administered, it effectively mimics the ileal-brake mechanism.

Literature on GLP-1 or a GLP-1-like peptides in fish is very sparse. Initially, a GLP was identified in the anglerfish and later the channel catfish (Plisetskaya *et al*. (1986). Later, Plisetskaya *et al*. (1986) isolated and determined the structure of Coho salmon (*Oncoryhnchus kisutch*) glucagon-like peptide. They showed it to be very similar to both anglerfish and catfish, with only two amino substitutions.

However, no work has been presented on the involvement of GLP-1 or GLP-1-like peptide on smooth muscle contractility or its potential involvement in gastric evacuation in fish at the time of writing. Therefore, in order to evaluate the effects of GLP-1 on the Chinook salmon GI tract and investigate it as a potential humoral factor involved in the Iintestinal brake hypothesis through a potential overproduction of the molecule, spontaneously contracting CS, PS, PSp and Int circular smooth muscle strips were challenged systematically by synthetic human GLP-1.

3.2.3.4 Serotonin (5-HT)

Serotonin (5-hyroxytryptamine) is an amine neurotransmitter that lacks a catechol group, which is synthesised from the amino acid tryptophan. It is found in high concentrations in the nervous tissue of vertebrates (Withers, 1992). And since the GI system is under both neural and humoral control, gastric evacuation is potentially partially controlled by 5-HT mediated neural feedback (Torsoli and Severi, 1993). It may cause contractions and/or

relaxations in multiple parts of the GI tract (Torsoli and Severi, 1993). Therefore, 5-HT was investigated in terms of its contractility in GI circular smooth muscle.

5-HT typically acts upon cell surface receptors, usually folded through the membrane in seven helices (Olsson and Holmgren, 2001). These seven-transmembrane-domain receptors bind to a G-protein, which in turn activates or inhibits enzyme cascades depending on the subtype of receptor and the subtype of G-protein (Olsson and Holmgren, 2001). There are numerous classes and subclasses of 5-HT receptors, involving different intracellular mechanisms. The most important receptors involved in smooth muscle contraction identified in the mammalian GI tract are 5-HT_{2a} with 5-HT_1 likely to play a lesser role (Woollard *et al.* 1994). Sanger and McClelland (1986) have shown endogenous 5-HT stimulation by 5-hydroxy-L-tryptophan causes cholinergically mediated longitudinal muscle contraction in the rat stomach. Similarly, Bucheit and Buhl (1994) have shown 5-HT to contract guinea pig stomach circular smooth muscle *in vitro.*

No fish 5-HT receptors have been isolated and sequenced at the time of writing. However, a 5-HT_{2a}-like receptor has been suggested to cause contraction in the rainbow trout intestine (Olsson and Holmgren, 2001). The contractile response to 5-HT was probably due to direct action upon the smooth muscle (Olsson and Holmgren, 2001). In contrast, the effect of 5-HT in several other teleosts may be partly mediated via stimulation of motor neurons, presumed to be cholinergic (Jensen and Holmgren, 1994).

Grove *et al.* (1974) demonstrated concentration-dependent 5-HT stimulation of plaice stomach longitudinal smooth muscle between $1.8x10^{-7}$ M and $5.6x10^{-7}$ M. Later, Cimini *et al*. (1985) showed 5-HT immunoreactive nerves in all layers of the dogfish stomach. Based on these data, it was concluded that 5–HT is highly likely to play a role

in Chinook GI smooth muscle activity in multiple locations in the GI tract. Therefore, in order to evaluate the effects of 5-HT on the Chinook salmon GI tract and investigate it as a potential humoral factor involved in the intestinal brake hypothesis through a potential overproduction of the molecule, spontaneously contracting CS and PSp circular smooth muscle strips were challenged concentration-dependently by synthetic 5-HT.

3.2.4 Intestinal Brake Humoral Dysfunction: a Process of GDAS?

The intestinal brake hypothesis predicts a post-prandial stimulus of a humoral factor/s or a neural stimulus dysfunction (Chapter 1). In order to investigate whether this was true, a study was initiated into the effects of serum collected from a GDAS affected (+ve) fish on isolated GI smooth muscle rings from unaffected Chinook salmon, with GDAS –ve fish serum acting as a control. Based on the literature, it was hypothesised that a humoral factor was the most likely candidate for the control of the intestinal brake in fish. In order to test this hypothesis, serum was collected from GDAS +ve and -ve fish raised in commercial sea-cage culture. The serum was diluted and applied to GI circular smooth muscle rings and the response recorded with a myograph. It was hoped a response indicative of an overactive intestinal brake response would be elicited.

3.2.5 Using Exogenous Hormones and Problems with Assays for Peptide GI Hormones

I used mammalian hormones, given the commercial unavailability of those occurring in fishes. Although the use of non-native peptides is undesirable, the logistics and time associated with the isolation of the native peptides were outside the scope of this thesis. To complicate matters, with the exception of gastrin, reproducible values of fasting and

post-prandial hormone levels in humans had not been obtained, and some like CCK still cannot be assayed with confidence due to the structural similarity of CCK forms and with the gastrin family (Ruppin and Domschke, 1980). CCK8 and gastrin1 show 90% cross-reactivity in RIA studies (Sigma, Australia). In addition, only human GLP-1 assays are commercially available and may be of little use in non-mammalian vertebrates. An assay for serum levels of 5-HT would also be futile, due to the ubiquitous nature of the molecule in vertebrate tissues (Withers, 1992). Therefore, no assays were performed in this study. Instead, myographical recordings from pharmacological application of the above mentioned factors was deemed the most sensible option for examining the biological activity in the Chinook GI tract.

3.2.6 Gastrointestinal Peptide and Neurotransmitter Study

Identification or isolation of factors present in serum or plasma poses a significant challenge (Matty, 1985). The identification of any humoral factor or neurotransmitter from serum generally requires a specific biological assay for the molecule (Matty, 1985). Alternatively, isolation of unknown structures can be attempted, and can be achieved by a number of methods if concentrations are high enough. One such method is by the use of nuclear magnetic resonance (NMR) technology. Most identification studies have prior knowledge of the factor to be isolated. When this factor is unknown, a diverse array of assays may be required, with a large amount of guess work involved. Even if the molecule to be assayed for is known, the assay may show high cross-reactivity. For example CCK8 shows 90% cross-reactivity with gastrin-1 when radioimmunoassayed (Sigma, Australia). The ‘blind’ identification of an unknown humoral factor by assay,

although not futile, can take vast amounts of time and resources. Therefore, due to the lack of feasibility of conducting a wide range of biological assays and the huge cost associated with this, the isolation of this/these factor/s was not attempted during this masterate study. Instead, based upon a literature review, the factors deemed most likely to show potent effects on the control of gastric evacuation and motility in fish (CCK8, gastrin-1, GLP-1 and 5-HT) were investigated in terms of their effects of isolated circular smooth muscle *in vitro*. These factors were hoped to exhibit patterns of activity on GI smooth muscle consistent with events known to occur during the intestinal brake in fish.

3.3 Materials and Methods

3.3.1 Sources of Fish

Freshwater Chinook salmon smolt were either obtained from NIWA's Silverstream hatchery (Canterbury) or Isaac's Salmon Farm (Canterbury). These fish were transported in aerated ice-chilled fish boxes to the Physiology Laboratory in the School of Biological Sciences, at the University of Canterbury. Upon arrival, the fish were either killed by stunning or kept in the University aquarium until needed.

3.3.2 Hormones

Sulphated CCK8, human gastrin-1, GLP-1 (36 amino fragment) 5-HT (all Sigma, Australia) were received dry and dissolved into distilled water to make up stock solutions of $1x10^{-1}$ M. These were frozen at -80˚C until needed for the experiments. Dilution series were made up before each experiment; CCK8 $1x10^{-9}$ M - $3x10^{-7}$ M; Gastrin-1 $1x10^{-13}$ M – $1x10^{-7}$ M, GLP-1 $1x10^{-13}$ M – $1x10^{-7}$ M; and 5-HT $1x10^{-13}$ M – $1x10^{-6}$ M. Preliminary experiments revealed that CCK8 between the concentrations of $1x10^{-13}$ M and $1x10^{-10}$ M produced no response in any preparation. As a consequence, these concentrations were excluded from further experiments. All treatments were applied to isolated tissue under tension in a myography preparation. All hormones were added in cumulative concentrations with a 15-minute period between additions.

3.3.3 Myography

Isolated CS, PS, PSp and Int rings were pharmacologically challenged with gastrin 1, CCK8, GLP-1 and 5-HT in separate experiments. Using myography, amplitude of the resultant response in milligrams contractile response and frequency of contraction of GI circular smooth muscle were measured. Fish were killed by stunning and subsequently incised along the ventral side of the abdomen from the gills to the anus. The whole GI tract was then dissected out and the CS, PS, PSp and Int isolated. CS rings were cut c.10mm from the end of the oesophageal sphincter. The pyloric stomach rings were cut c.10mm caudal to the end of 'J' bend in the stomach. The pyloric sphincter was removed at the junction between the stomach and intestine. The intestinal rings were cut from the region caudal to the pyloric caecae. These were stored in freshwater salmon Ringer (FSR, Chapter 2) at 10˚C until used. GI smooth muscle rings were 'strung-up' between a fixed point and a calibrated Powerlab™ pressure transducer, using surgical stitching silk. The preparation was bathed in 20ml of aerated salmon Ringer at 10˚C. Following preliminary testing, tissues were tensioned in the optimum range (so as not to under-tension or damage the tissue) and allowed to rest and begin spontaneous contractions. Hormones were added cumulatively to the Ringer baths and the trace of the tension developed recorded. Each piece of tissue was challenged only one cumulative concentration series. Powerlab™ output was fed directly into a HP Compaq nx 9040 laptop running Microsoft Chart 5™, where an instantaneous analog-digital signal was recorded to disc. Contraction force was recorded in milligrams (mg). After the experiment, all tissue samples were blotted dry and weighed to 4dp. See photograph 1 overleaf showing experimental setup.

Photograph 1. Myography setup showing Powerlab® and laptop to the left and custom built myograph to the right.

3.3.4 Serum Study

GDAS +ve blood was collected from fish affected by the syndrome. These fish were harvested from commercial sea-cages in the Marlborough Sounds, New Zealand. Control blood was collected from fish from the same population that were not affected by GDAS. GDAS assessment of fish was based on the New Zealand King Salmon Company's criteria (similar to the simplified assessment described in Chapter 2). Blood was collected via caudal puncture, allowed to clot and centrifuged (10,000 rpm for 10 mins) in an Eppendorf ™ centrifuge. The serum was pipetted off into a clean 1.5ml Eppendorf ™ tube. The samples were subsequently frozen and stored at -80˚C until needed. Later, the serum was administered to isolated GI tissues, prepared in the exact same manner as the other myography conducted in the current study, at 1000x and 100x dilutions (n=6 for

each concentration). After being exposed to a 1000-fold dilution for fifteen minutes, the Ringer bath serum concentration was cumulatively increased to make up a 100-fold serum dilution. However due to a limited supply of serum, only CS and PSp circular smooth muscle rings were used for these experiments as these tissues were deemed to be the most informative parts of the GI tract when considering the intestinal brake.

3.3.5 Data Analysis

All data were standardised to wet tissue mass and tested for normality in Microsoft Minitab® using the normality plot function before statistical testing. All datasets used were determined to have Gaussian distributions, on two occasions log10 transformation was required to normalise datasets. Two-tailed unpaired Student's t-tests were used to analyse the serum study data. While sigmoidal concentration-response curves were fitted to the myography data and differences in PFs and EC50s determined by two-tailed unpaired Student's t-tests. . Linear and 2nd order polynomial regression lines were fitted to the SPM data and significance determined by F-tests on the departure of the regression lines from zero. For the concentration-response curves, both log 10 EC_{50} and EC_{50} were calculated. Log 10 EC_{50} was used for comparisons. Anti-log10 of this value is shown on the figures for comparative purposes. Analysis was performed in Microsoft Chart 5®; Excel®; Graphpad Prism 4® and Minitab®.

3.3.6 Animal Ethics

Animals were killed by stunning rather than anaesthesia, due to the known relaxant effect of anaesthetic on the smooth muscle of experimental fish (Hill *et al.* 2004). All

experiments conducted were approved by the University of Canterbury Animal Ethics Committee.

3.4 Results

3.4.1 GDAS +ve Serum Effects on GI Smooth Muscle

Serum from GDAS +ve fish contracted stomach circular smooth muscle. Comparisons made between the effects of GDAS +ve serum and controls (GDAS -ve) on TMS change (Δ) in peak net force generated by GI smooth muscle revealed that GDAS +ve serum produced a significantly increased tonus of the cardiac stomach at a 1000-fold (P=0.0016) and 100-fold dilution (P=0.0497) reaching a mean ± SEM peak rise in tension of 0.073 ± 0.004 $mg.mg^{-1}$ and 0.116 ± 0.041 $mg.mg^{-1}$ respectively, after 15 minutes (Figure 3.1). Drift corrected raw traces show the Δ in net force (mg) with a 1000-fold (Figure 3.2) and 100-fold (Figure 3.3) dilution of the serum. Similarly, GDAS +ve serum produced a significantly increased tonus of the pyloric sphincter at a 1000-fold (P=0.0003) and 100-fold (P=0.0169) dilutions reaching a mean ± SEM peak tension rise of 0.221 ± 0.037 $mg.mg^{-1}$ and 0.256 ± 0.083 $mg.mg^{-1}$ respectively after 15 minutes (Figures 3.4). Drift corrected raw traces show Δ in net force (mg) with a 1000-fold (Figure 3.5) and 100-fold (Figure 3.6) dilution of the serum.

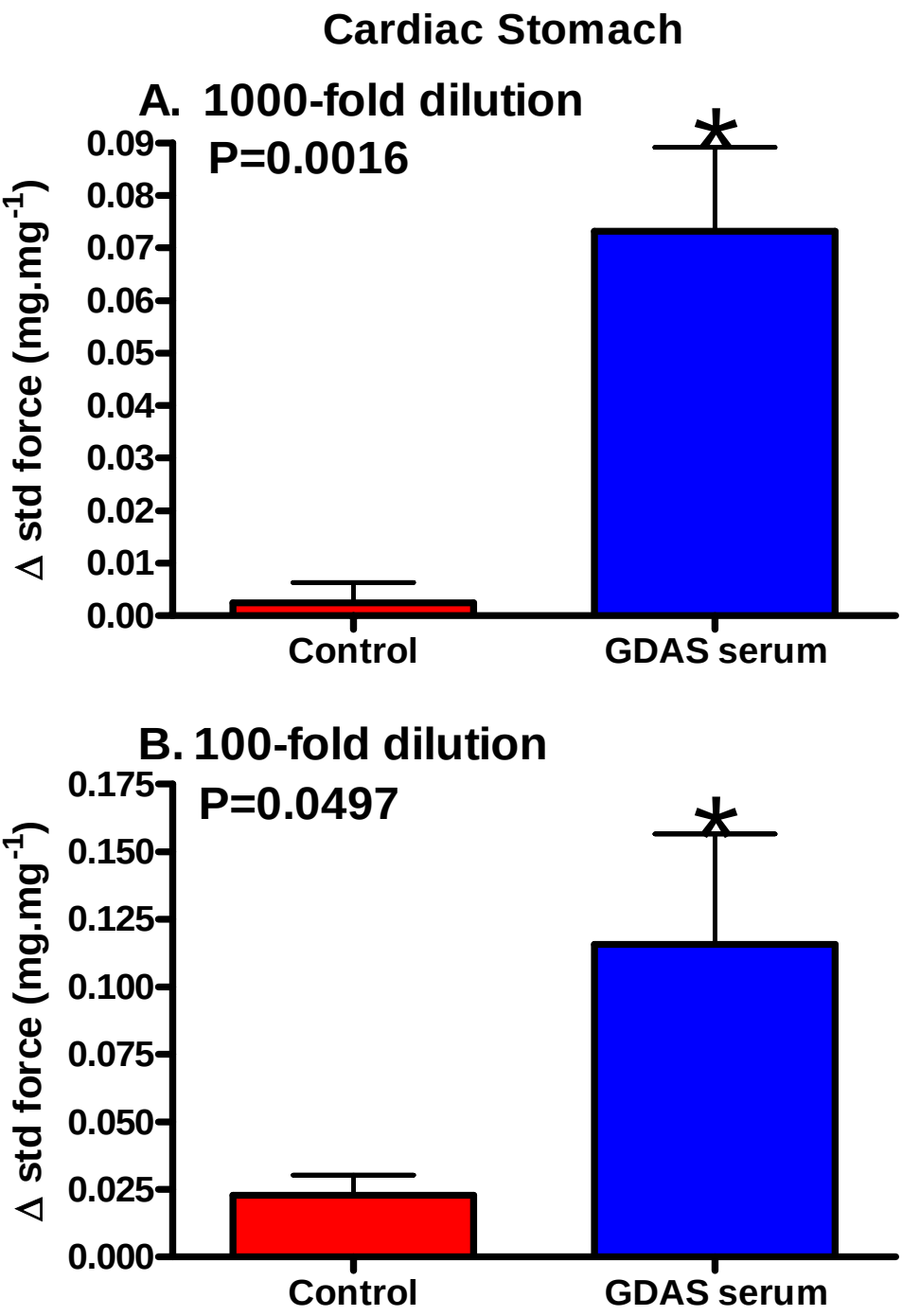

Figure 3.1. Wet mass standardised Δ in net force ($mg.mg^{-1}$), generated by cardiac stomach circular smooth muscle rings challenged by GDAS +ve (blue) and control (red) serum, after 15 minutes *in vitro*. **A.** standardised forces generated by a 1000-fold serum dilution. **B.** standardised forces generated by a 100-fold serum dilution (note different scales). ***** represents significant differences calculated by two-tailed unpaired Student's t-tests. Data are mean ± SEM. n=8 for each treatment/dilution combination.

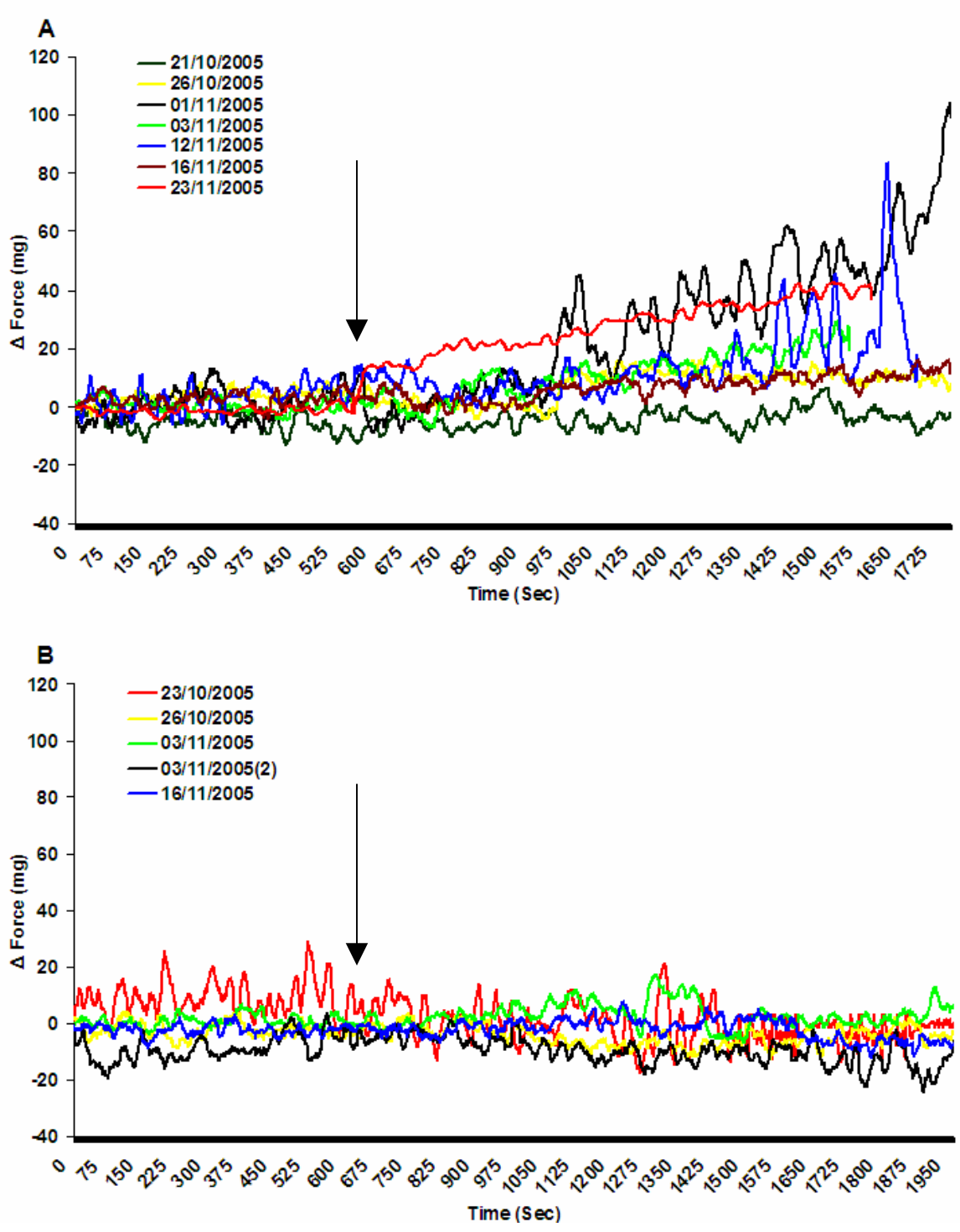

Figure 3.2. Drift corrected representative raw traces showing the Δ in net force (mg) generated by cardiac stomach circular smooth muscle rings pharmacologically challenged by GDAS +ve (**A**) and control (**B**) Chinook salmon serum diluted 1000-fold. Arrows indicate the addition of serum into the Ringer baths.

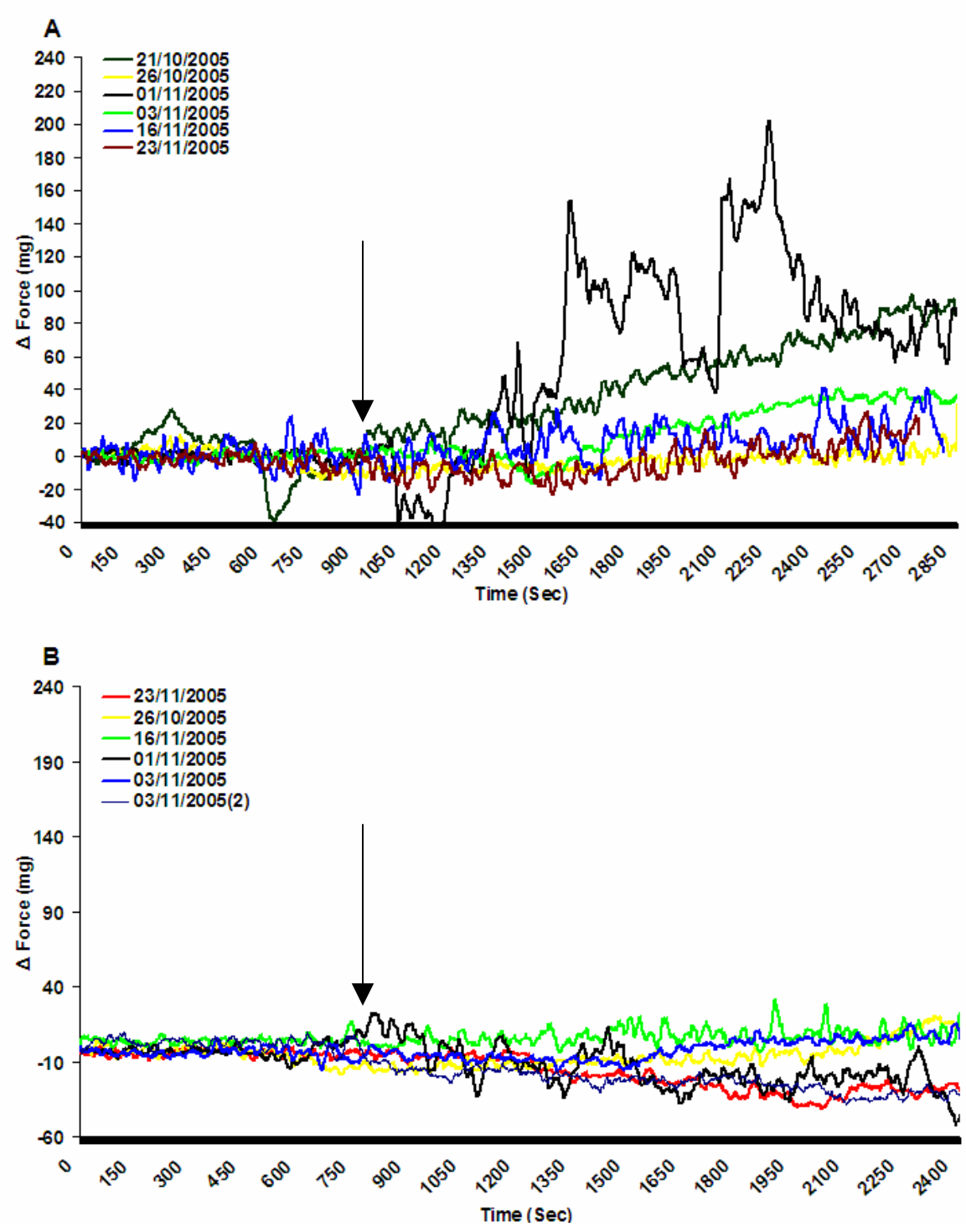

Figure 3.3. Drift corrected representative raw traces showing Δ in net force (mg) generated by cardiac stomach circular smooth muscle rings pharmacologically challenged by GDAS +ve (**A**) and control (**B**) Chinook salmon serum diluted 100-fold. Arrows indicate the addition of serum into the Ringer baths.

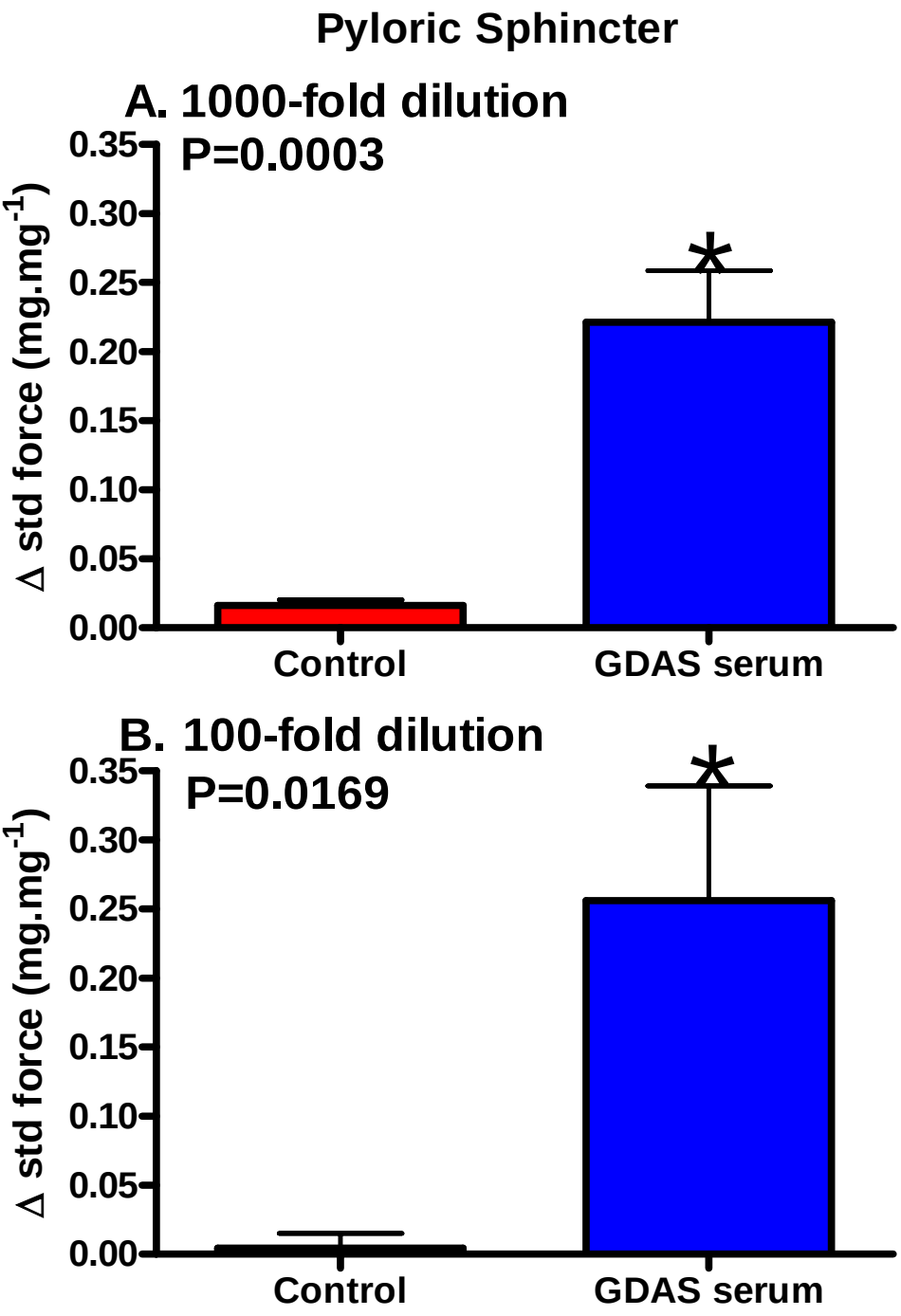

Figure 3.4. Wet mass standardised Δ in net force (mg.mg^{-1}), generated by pyloric sphincter circular smooth muscle rings challenged by GDAS +ve (blue) and control (red) serum, after 15 minutes, *in vitro*. **A.** standardised forces generated by a 1000-fold serum dilution. **B.** standardised forces generated by a 100-fold serum dilution (note different scales). ***** represents significant differences calculated by two-tailed unpaired Student's t-tests. Data are mean ± SEM. n=8 for each treatment/dilution combination.

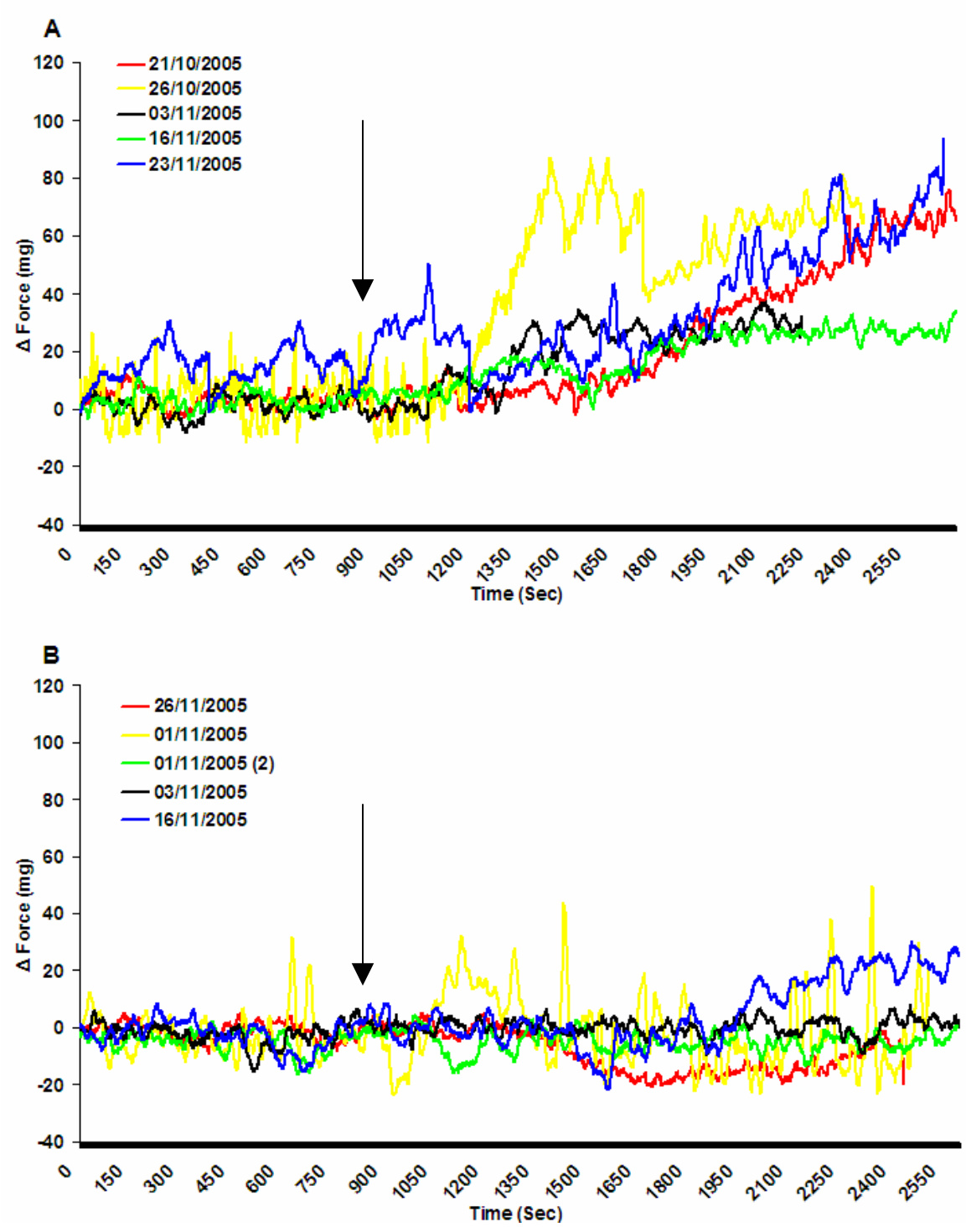

Figure 3.5. Drift corrected representative raw traces showing Δ in net force (mg) generated by pyloric sphincter circular smooth muscle rings pharmacologically challenged by GDAS +ve (**A**) and control (**B**) Chinook salmon serum diluted 1000-fold. Arrows indicate the addition of serum into the Ringer baths.

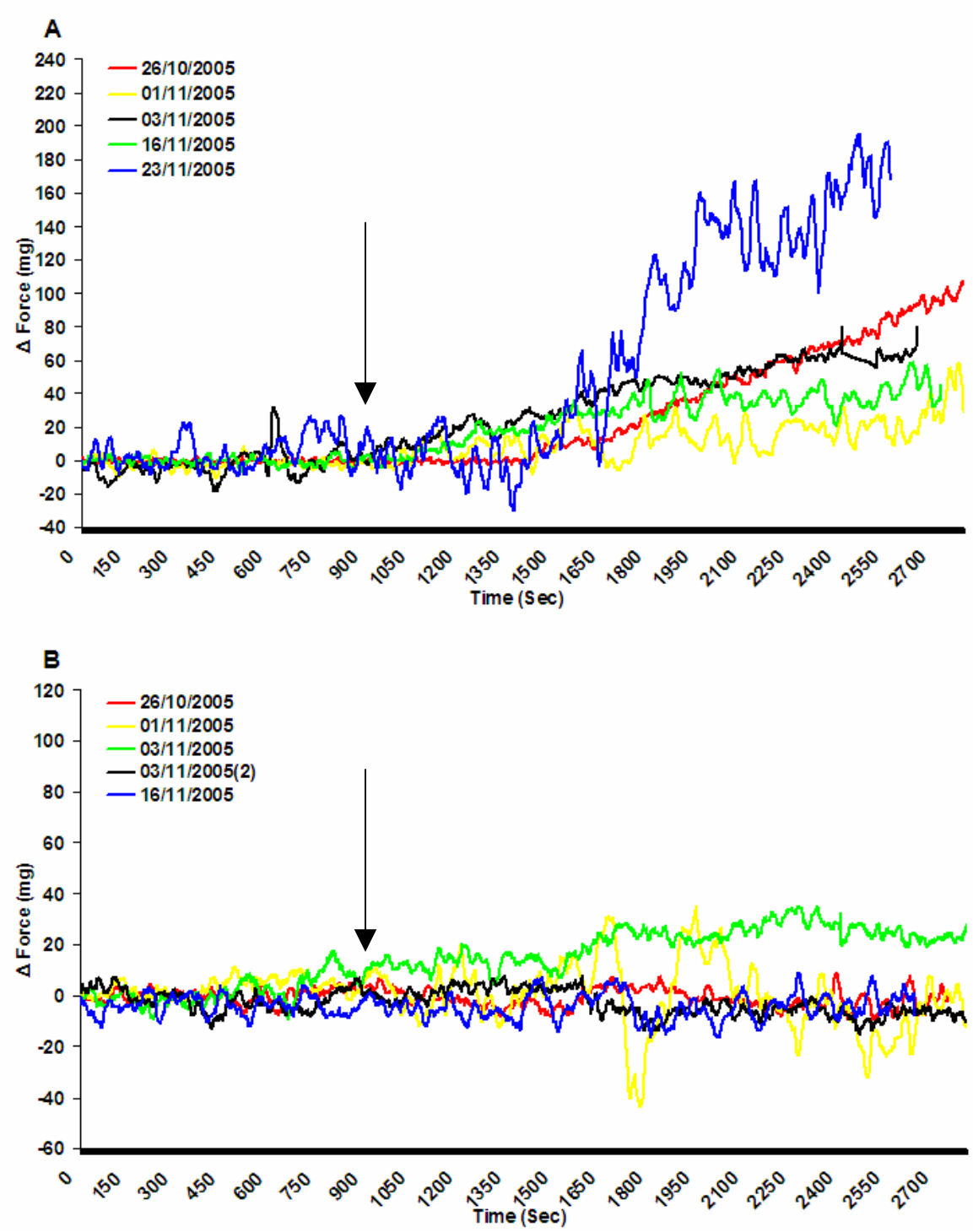

Figure 3.6. Drift corrected representative raw traces showing Δ in net force (mg) generated by pyloric sphincter circular smooth muscle rings pharmacologically challenged by GDAS +ve (**A**) and control (**B**) Chinook salmon serum diluted 100-fold. Arrows indicate the addition of serum into the Ringer baths.

3.4.2 Gastrointestinal Peptide Study

3.4.2.1 Cholecystokinin-8 (CCK8)

Concentration-dependency of Chinook salmon GI circular smooth muscle contractility, in terms of wet tissue mass standardised (TMS) peak net force ($mg.mg^{-1}$), was demonstrated in response to CCK8 in cardiac stomach (CS), pyloric stomach (PS), pyloric sphincter (PSp) and intestinal (Int) gut rings. Sigmoidal concentration-response curves were calculated for CCK8 in terms of TMS mean peak net force of spontaneous rhythmic contractions produced, with the force produced shown as a proportion of the maximal response in any preparation. Log10 molar effective concentration (EC_{50} ± 95% Confidence Interval (CI)), and mean peak force (PF) (± SEM), values were calculated for CCK8 in terms of TMS peak net force produced ($mg.mg^{-1}$). The values calculated for CCK8 (n=7) were; CS log10 EC_{50} -8.15 ± 0.90, PF 46.8 ± 13.2 (Figure 3.7A); PS log10 EC_{50} -7.88 ± 0.48, PF 32.2 ± 9.6 (Figure 3.8A); PSp log10 EC_{50} -8.98 ± 0.68, PF- (155.2 ± 59.5 (Figure 3.9A); Int log10 EC_{50} -8.93 ± 0.64, PF 69.0 ± 20.0 (Figure 3.10A). When a one-way ANOVA comparing log10 EC_{50}s from the four tissues was performed, it was found that there was no significant difference (P=0.2588) in the means. Tukey's post-hoc comparisons showed that none of the tissues were significantly different from each other.

Rate of spontaneous spike frequency per minute (SPM) also showed a concentration-dependent response to CCK8 in all tissues, except intestine. Linear regression of SPM in CS circular smooth muscle tissue revealed a significant negative relationship (P=0.0012, R^2=0.94) (Figure 3.7B). Linear regression of the PS SPM fitted the data so poorly that the decision was made to use polynomial regression for analysis. Second-order polynomial regression of SPM in PS circular smooth muscle tissue

revealed a positive hyperbolic relationship (R^2=0.90) (Figure 3.8B). Linear regression of SPM in PSp circular smooth muscle tissue revealed a significantly positive relationship (P=0.0132, R^2=0.82) (Figure 3.9B). Linear regression of SPM in Int circular smooth muscle tissue showed no significant relationship (P=0.5577, R^2=0.09) (Figure 3.10B).

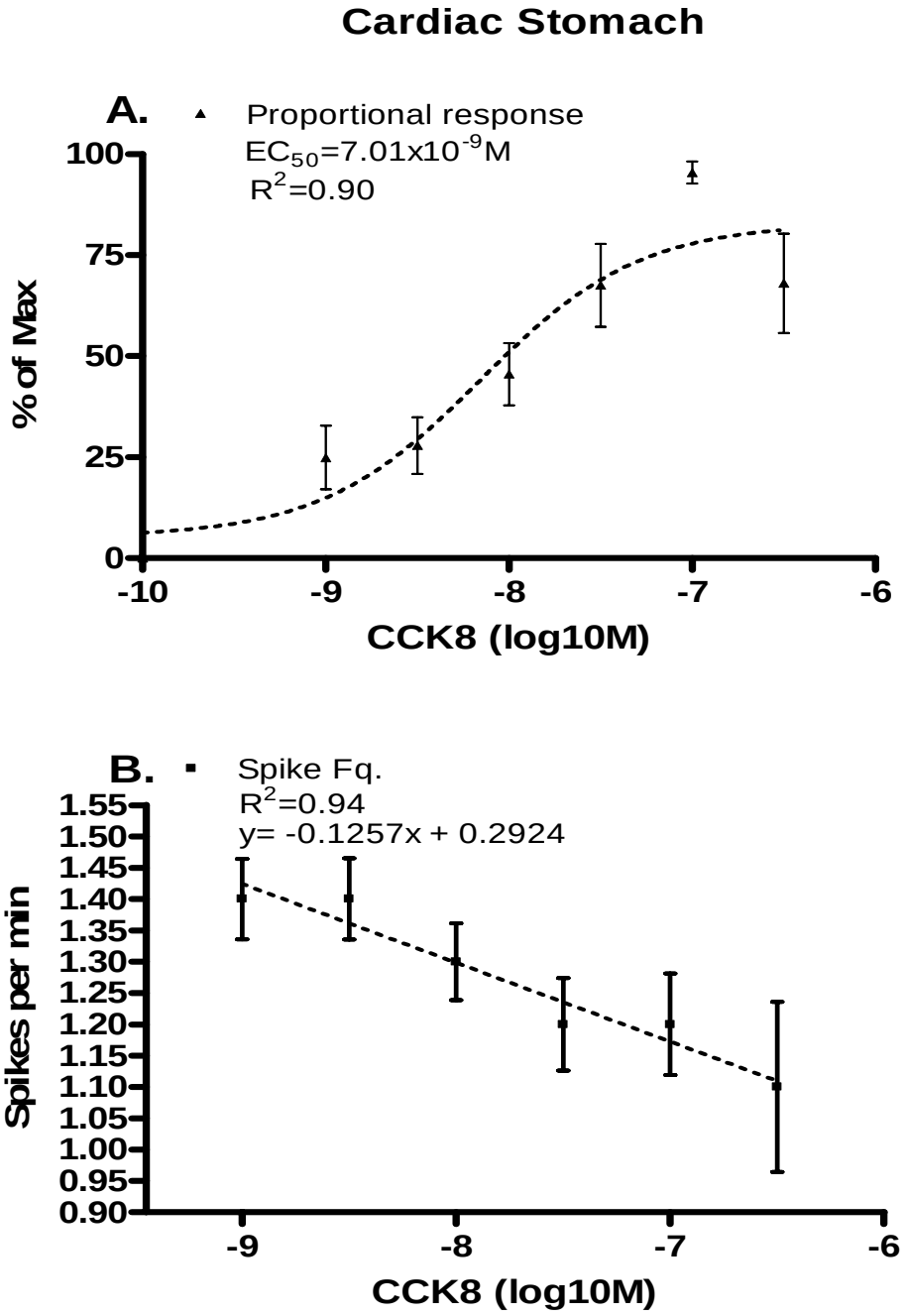

Figure 3.7. CCK8 pharmacology on isolated Chinook salmon cardiac stomach circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Negative linear regression best describes the data (r^2=0.94) which is significantly non-zero (P=0.0012). Both graphs show responses to CCK8 between the concentrations of $1x10^{-9}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

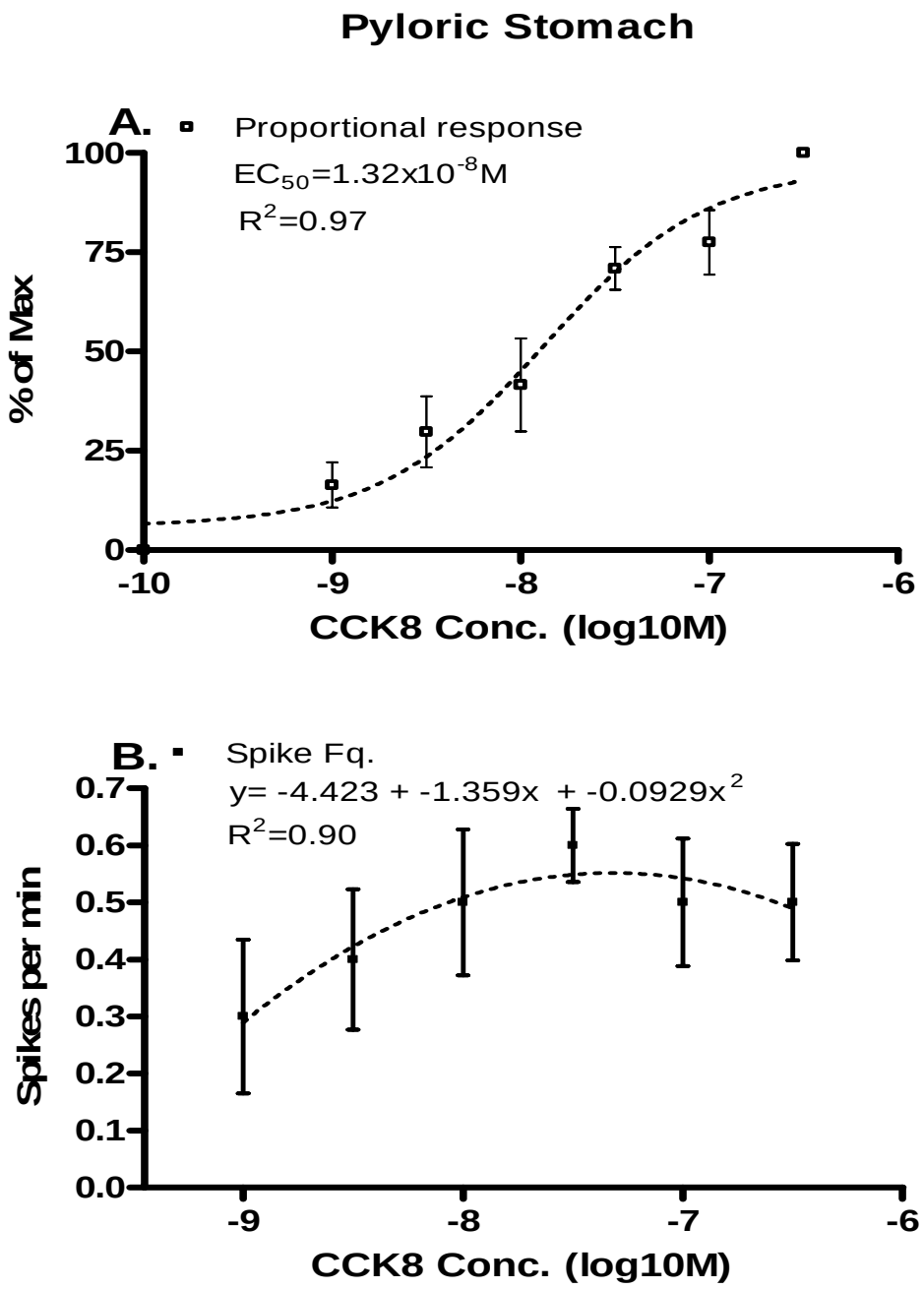

Figure 3.8. CCK8 pharmacology on isolated Chinook salmon pyloric stomach circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Second order polynomial regression best describes the data (r^2=0.90). Both graphs show responses to CCK8 between the concentrations of $1x10^{-9}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

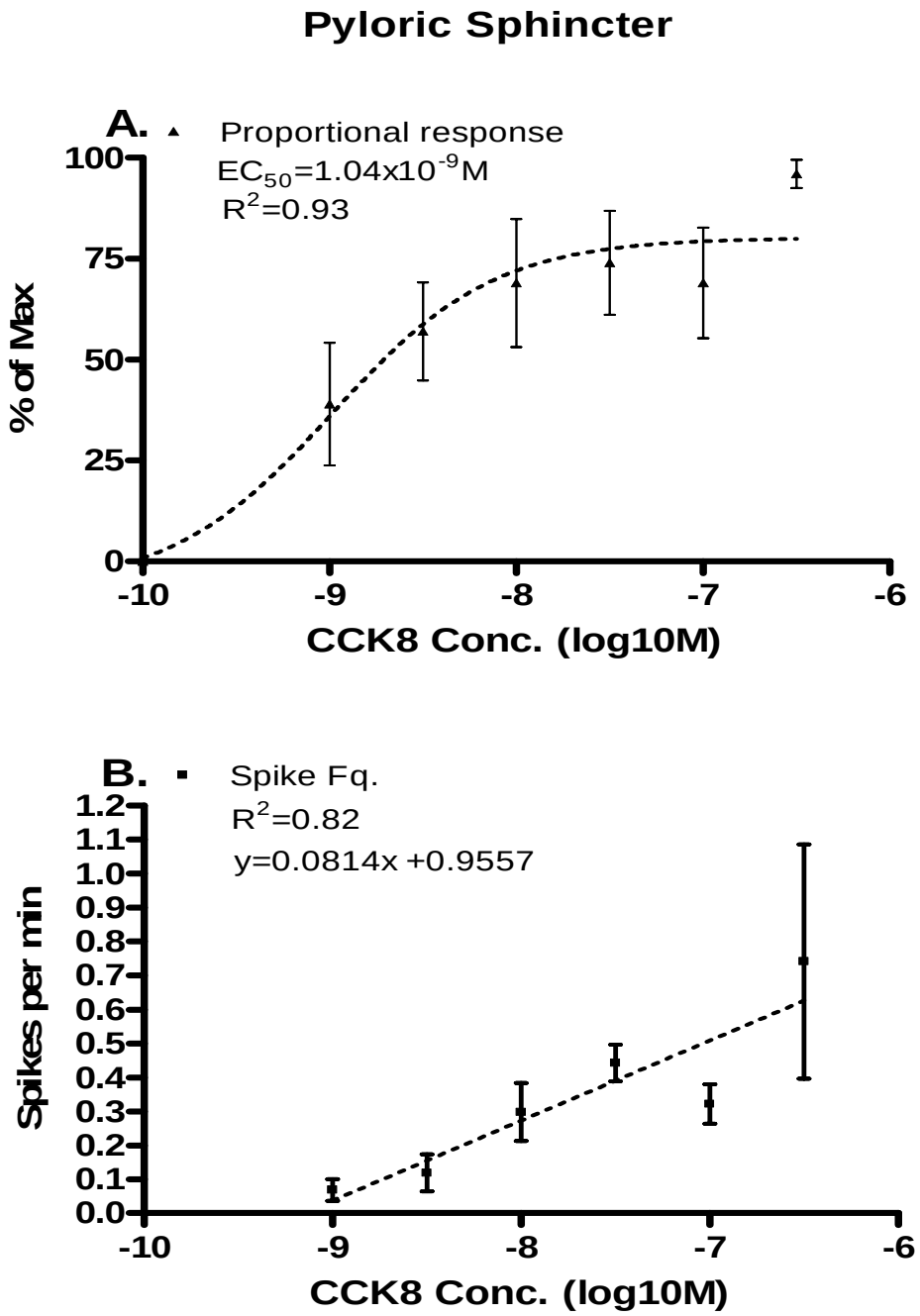

Figure 3.9. CCK8 pharmacology on isolated Chinook salmon pyloric sphincter circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Positive linear regression best describes the data (r^2=0.82) which is significantly non-zero (P=0.0132). Both graphs show responses to CCK8 between the concentrations of $1x10^{-9}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

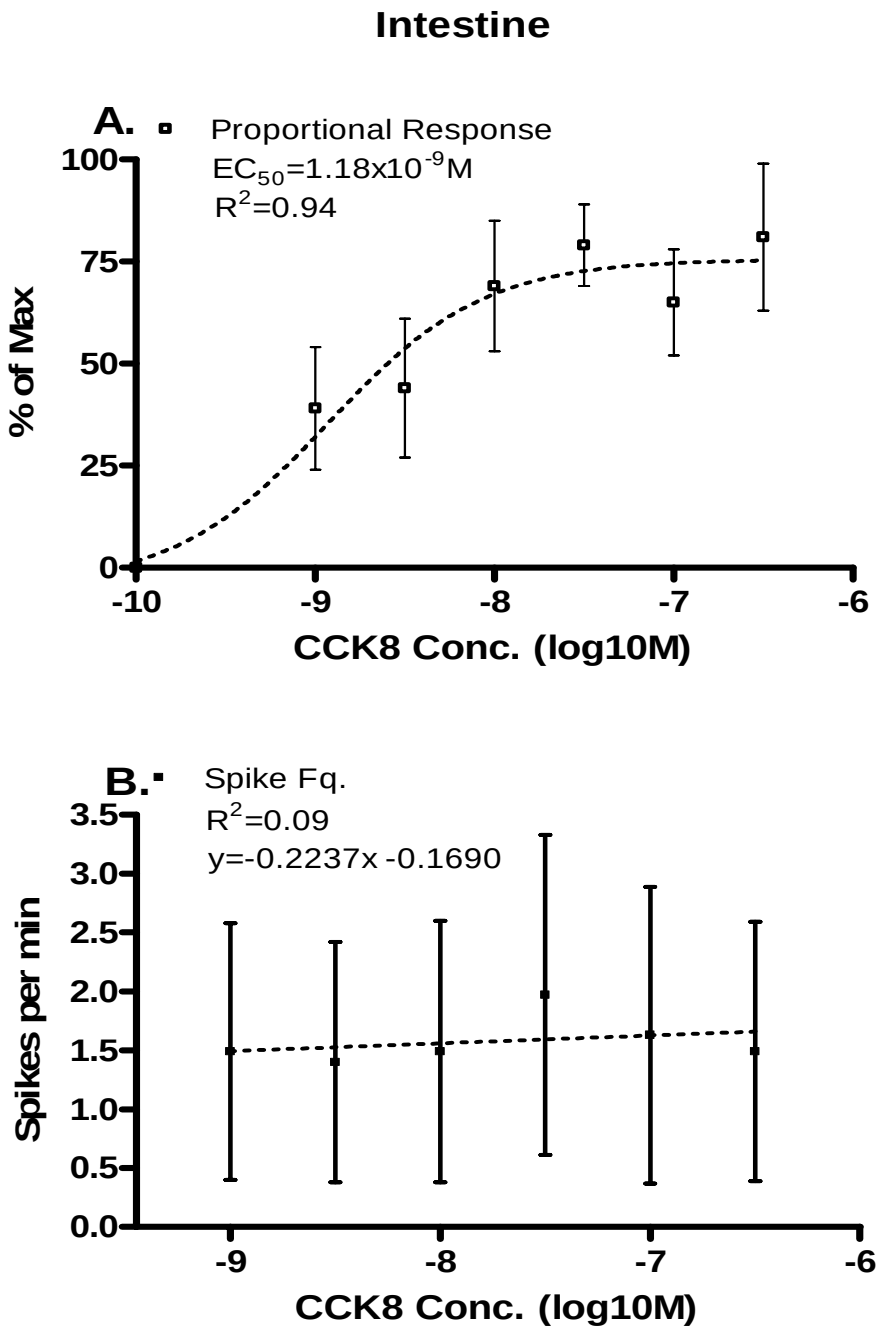

Figure 3.10. CCK8 pharmacology on isolated Chinook salmon intestinal circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Linear regression poorly describes the data (r^2=0.09), which is not significantly non-zero (P=0.5577). Both graphs show responses to CCK8 between the concentrations of $1x10^{-9}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

3.4.2.2 Gastrin-1

As with CCK8, concentration-dependency of Chinook salmon GI circular smooth muscle contractility, in terms of TMS peak net force ($mg.mg^{-1}$), was demonstrated in response to gastrin-1 in CS, PS, PSp and Int gut rings. Log10 EC_{50} ± CI, and mean PF ± SEM, values were calculated for gastrin-1 in terms of TMS peak net force produced ($mg.mg^{-1}$). The values calculated for gastrin-1 (n=7) were; CS log10 EC_{50} -12.45 ± 0.66, PF 36.3 ± 12.6 (Figure 3.11A); PS log10 EC_{50} -12.55 ± 0.63, PF 42.5 ± 1.0 (Figure 3.12A); PSp log10 EC_{50} -9.35 ± 0.78, PF 162.0 ± 56.3 (Figure 3.13A); Int log10 EC_{50} -12.69 ± 1.12, PF 56.0 ± 10.9 (Figure 3.14A). When a one-way ANOVA comparing log10 EC_{50}s from the four tissues was performed, it was found that there was a significant difference ($P<0.0001$) in the means. Tukey's post-hoc comparisons showed that the PSp was significantly less sensitive than CS ($P=0.0016$), PS ($P=0.0014$) and Int ($P<0.0001$). Interestingly, an unpaired Student's t-test between PSp log10 EC_{50}s from CCK8 and gastrin-1 showed no significant difference ($P=0.4893$).

SPM also showed a concentration-dependent response to gastrin-1 in all tissues except Int. Linear regression of SPM in CS circular smooth muscle tissue revealed a weak, but significant negative relationship ($P=0.0423$, $R^2=0.47$) (Figure 3.11B). Linear regression of SPM in PS circular smooth muscle tissue revealed a significantly positive relationship ($P=<0.0001$, $R^2=0.88$) (Figure 3.12B). Linear regression of SPM in PSp circular smooth muscle tissue revealed a significantly positive relationship ($P=0.0004$, $R^2=0.89$) (Figure 3.13B). Linear regression of SPM in Int circular stomach smooth muscle tissue revealed a possible positive relationship which bordered on significance ($P=0.0611$, $R^2=0.53$) (Figure 3.14B).

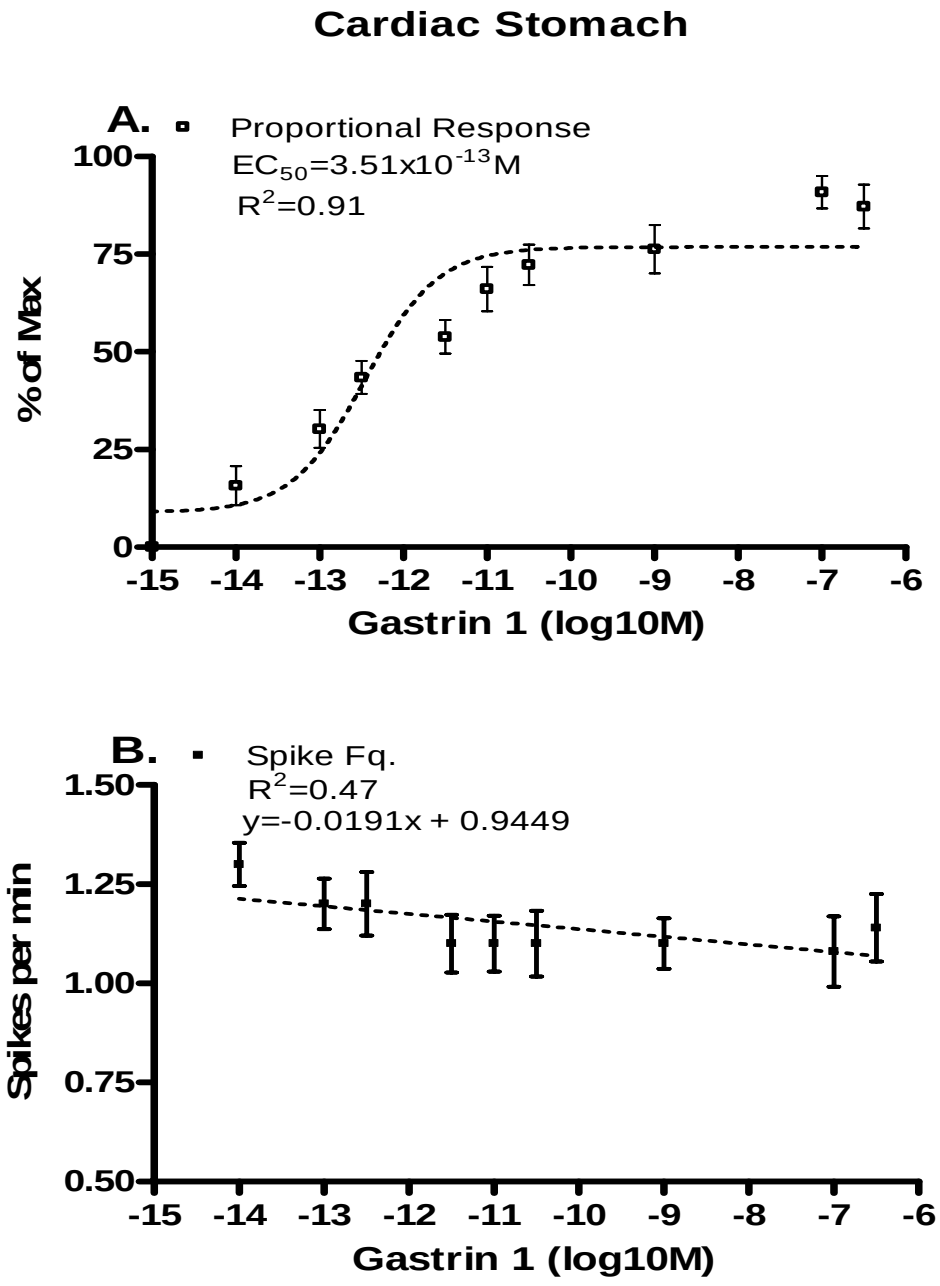

Figure 3.11. Gastrin-1 pharmacology on isolated Chinook salmon cardiac stomach circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Negative linear regression best describes the data (r^2=0.47) which is significantly non-zero (P=0.0423). Both graphs show responses to gastrin-1 between the concentrations of $1x10^{-14}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

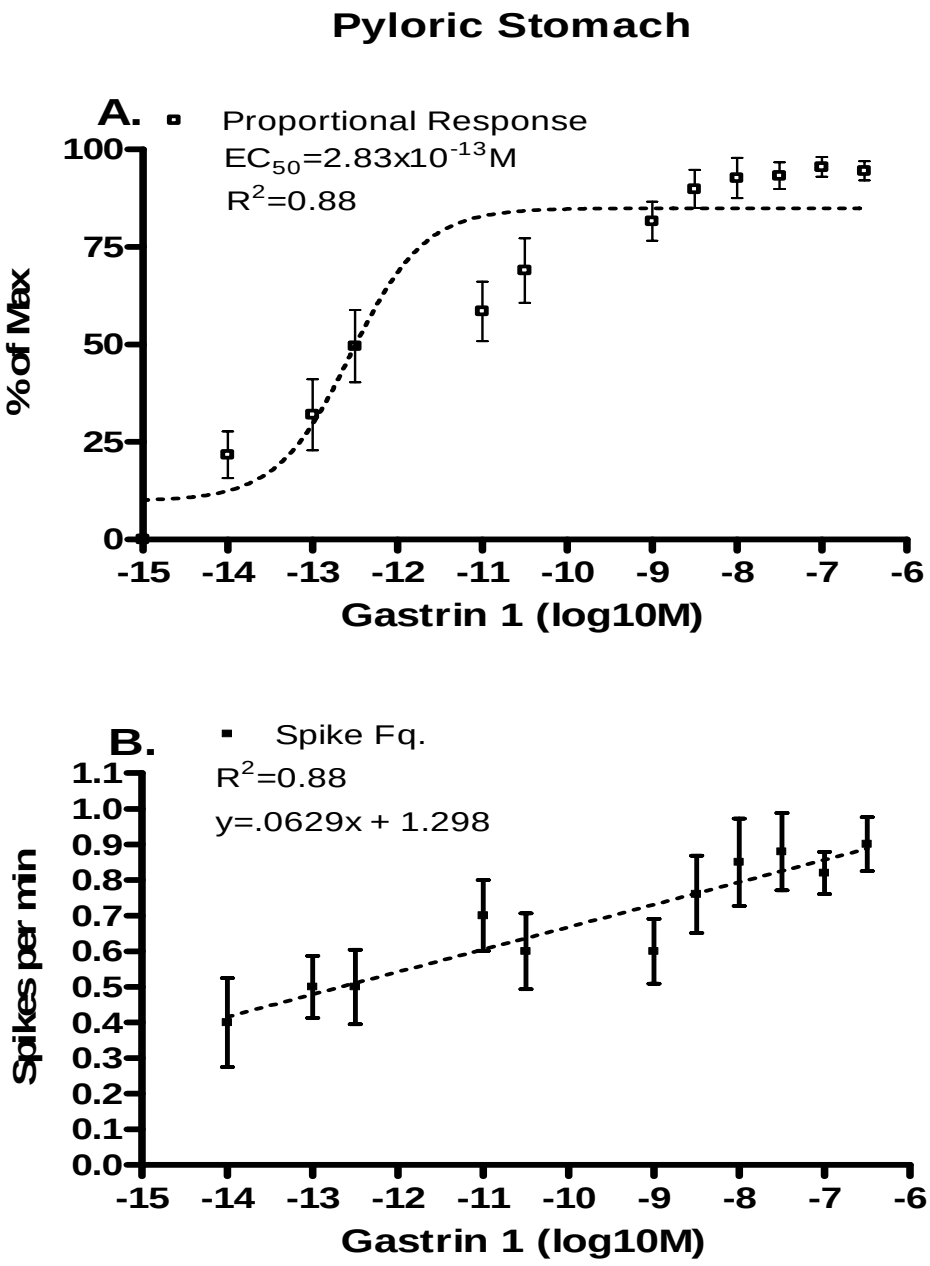

Figure 3.12. Gastrin-1 pharmacology on isolated Chinook salmon pyloric stomach circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Positive linear regression best describes the data (r^2=0.88) which is significantly non-zero (P=<0.0001). Both graphs show responses to gastrin-1 between the concentrations of $1x10^{-14}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

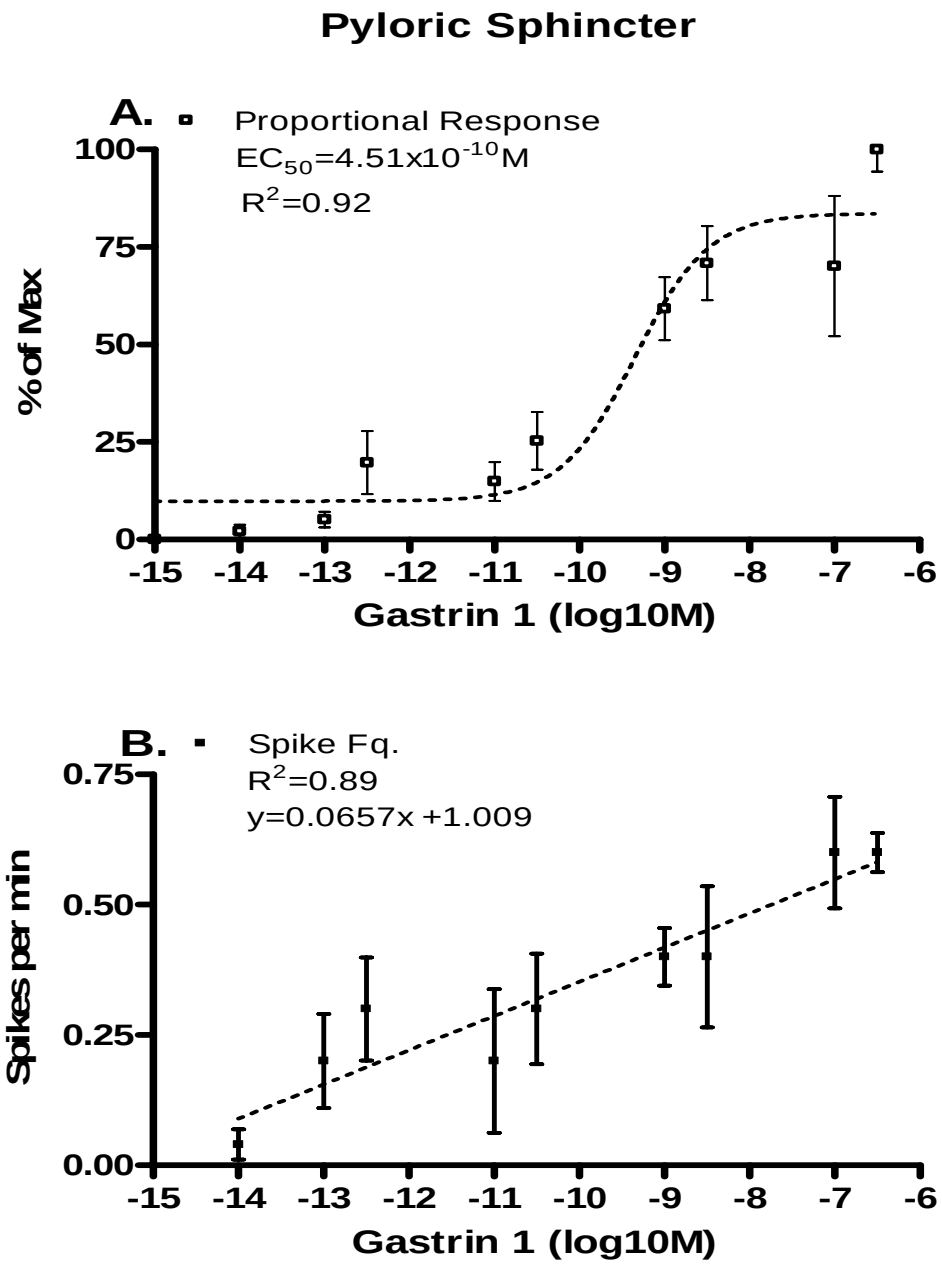

Figure 3.13. Gastrin-1 pharmacology on isolated Chinook salmon pyloric sphincter circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Positive linear regression best describes the data (r^2=0.89) which is significantly non-zero (P=0.0004). Both graphs show responses to gastrin-1 between the concentrations of $1x10^{-14}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

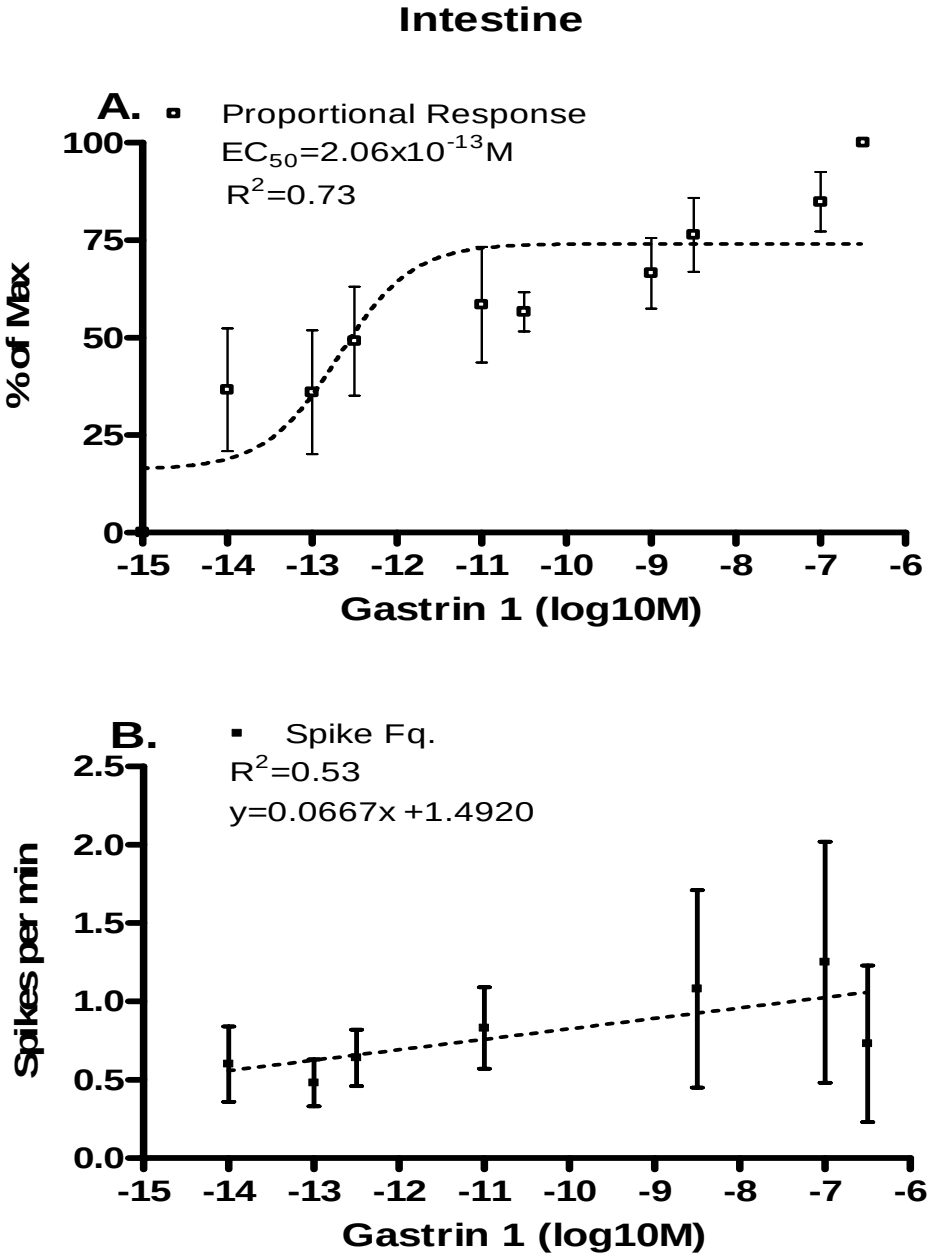

Figure 3.14. Gastrin-1 pharmacology on isolated Chinook salmon intestinal circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Positive linear regression best describes the data (r^2=0.53) which is not significantly non-zero (P=0.0611). Both graphs show responses to gastrin-1 between the concentrations of $1x10^{-14}$ and $3x10^{-7}$ M. All data are means ±SEM. n=8.

3.4.2.3 Serotonin (5-HT)

Due to time constraints only CS and PSp circular smooth muscle rings were pharmacologically challenged with 5-HT. These two tissues were deemed to be the most informative tissues to characterise, due to their known role in gastric motility (Ruppin and Domschke, 1980). Concentration-dependency of Chinook salmon GI circular smooth muscle contractility, in terms of TMS peak net force (mg.mg^{-1}), was demonstrated in response to 5-HT in CS and PSp gut rings. Log 10 EC_{50} and mean PF (± SEM) values calculated for 5-HT in terms of TMS peak net force produced (n=6) were; CS log10 EC_{50} -4.78 ± 1.05, PF 7.5 ± 2.1 (Figure 3.15A) and PSp log 10 EC_{50} -6.18 ± 1.14, PF 3.3 ± 1.2 (Figure 3.16A). It is worth noting that the responses elicited by Chinook GI tissues to 5-HT were weakly sigmoidal. This is similar to the strong sigmoidal responses generated in response to the peptides used in this study, although the concentration required to produce this response is much higher in absolute terms. An unpaired Student's t-test between log10 EC_{50}s from CS and PSp tissues showed that they were significantly different (P=0.0174).

SPM linear regression also showed a concentration-dependent relationship in both tissues subjected to 5-HT pharmacology. SPM in CS and PSp circular smooth muscle tissue revealed a significantly positive relationship CS: (P=0.0094, R^2=0.77) (Figure 3.15B), PSp (P=0.0103, R^2=0.76) (Figure 3.16B).

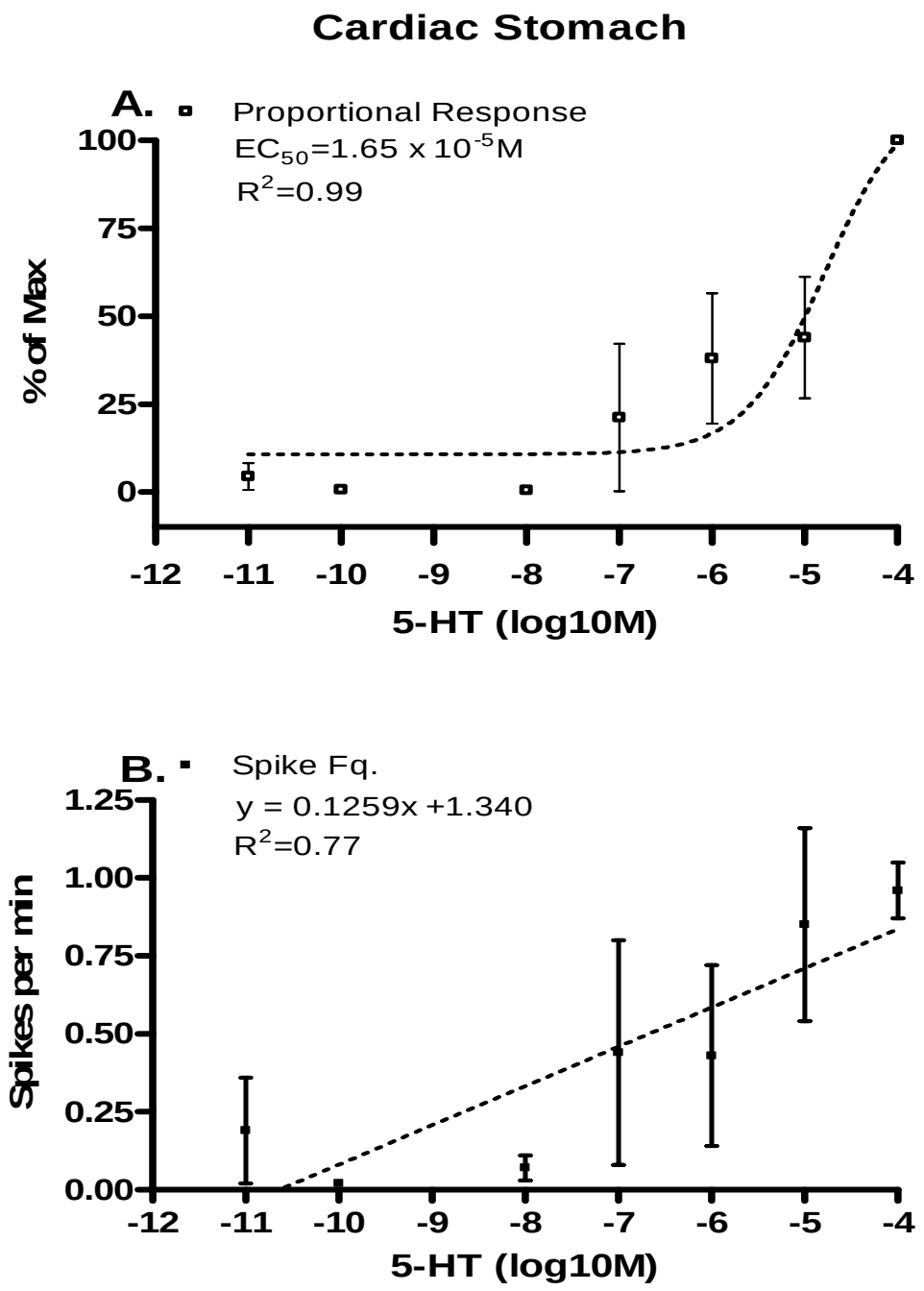

Figure 3.15. 5-HT pharmacology on isolated Chinook salmon cardiac stomach circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Positive linear regression best describes the data (r^2=0.77) and is significantly non-zero (P=0.0094). Both graphs show responses to 5-HT between the concentrations of $1x10^{-11}$ and $1x10^{-4}$ M. All data are means ±SEM.n=5.

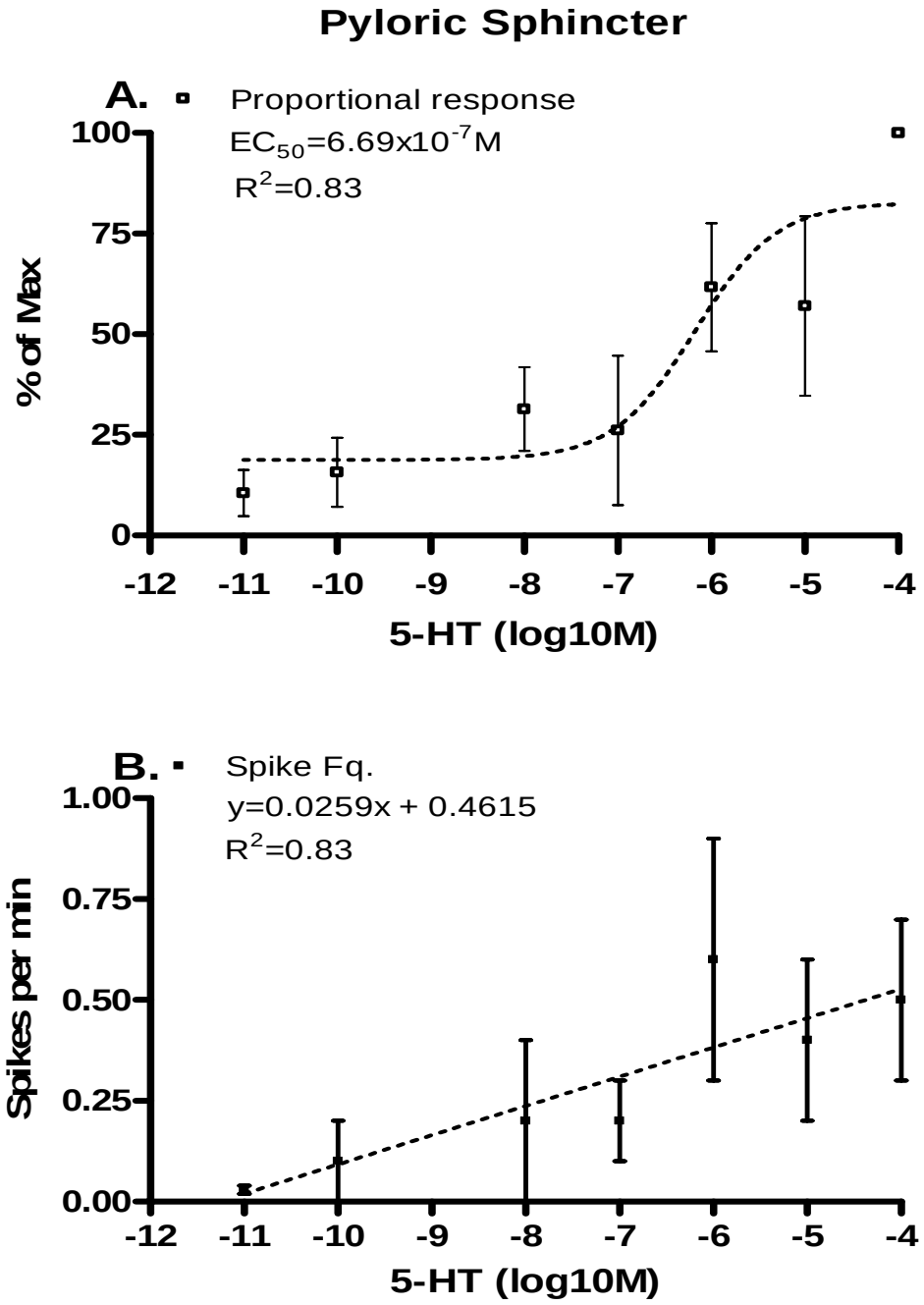

Figure 3.16. 5-HT pharmacology on isolated Chinook salmon pyloric sphincter circular smooth muscle rings. **A**. Concentration-response curve of the proportion of the maximal response (%) of the wet mass standardised peak net force of spontaneous rhythmic contraction. The EC_{50} value is shown. **B**. Spike rate per minute (SPM) of spontaneous rhythmic contraction. Positive linear regression best describes the data (r^2=0.76) which is significantly non-zero (P=0.0103). Both graphs show responses to 5-HT between the concentrations of $1x10^{-11}$ and $1x10^{-4}$ M. All data are means ±SEM. n=5.

3.4.2.4 Glucagon-Like-Peptide-1 (GLP-1)

GLP-1 (n=4) produced no response in any of the tissues examined between the concentration range $1x10^{-13}$ M – $1x10^{-6}$ M. Therefore, no further replicates were completed and no statistics were calculated.

3.5 Discussion

3.5.1 Gastric Motility Patterns Identified in Historical Studies: Correlates with the Current *in vitro* Study

In 1968, Alvarez wrote:

"Movements of the alimentary canal resulting in propulsion, segmentation and mixing of contents depend on the presence of contractile elements (smooth muscle) whose organisation and cellular structure can be correlated with the type of motor activity exhibited by the major subdivisions of the gut."

These subdivisions can show marked species variation but general function has been conserved over evolutionary time (Ganong, 1977). One such example is the stomach, which has the ability to retain digesta by co-ordinating the contractility of specific regions. In mammals and other vertebrate groups, peristaltic waves are known to propagate from the proximal end of the stomach to the distal portion. W. B. Cannon pioneered work on peristaltic wave propagation in vertebrates, working predominantly with cats at the end of the 19th century (Alvarez, 1968). Some of his work on this appeared in the first volume of the American Journal of Physiology in 1898, where, using X-rays, he reported that the propagation of peristaltic waves, originating from the upper curvature of the stomach, progress over the 'distal body' and antrum terminating in the pylorus (Davenport, 1989). Later work by Cannon and others formed the basis of our current understanding of GI motility in mammals. Their data showed that after ingesting a meal, peristaltic waves of the stomach terminate in a strongly contracted pyloric sphincter which prevents the movement of digesta into the duodenum, only periodically

allowing chyme through. Cannon described the PS as the 'keeper of the gate' and found that pyloric sphincter mediated gastric evacuation is directly related to the caloric value of the meal (Davenport, 1989). His work in 1911 using the cat GI tract as a model showed that 'carbohydrate' meals are emptied from the stomach slower that 'protein' meals, which are emptied faster than 'fat' meals (Davenport, 1989). This slowed emptying was accompanied by a slowing of peristaltic wave propagation speed and frequency. The control of the pyloric sphincter soon became a hot topic in GI research and was investigated by many. In the 1930s R. K. S. Lim showed humoral regulation of gastric motility (slowed peristalsis and gastric secretion and reduced stomach emptying) by a substance secreted in the duodenum. Lim named this hormone an 'enterogasterone' (Davenport, 1989). Shortly before his death 1950, B. P. Babkin presented his research into the excitory and inhibitory control of the pyloric sphincter in dogs via vagal afferent pathways (Davenport, 1989). It has long been known that both neural and humoral regulation is exerted upon GI tissues to produce slowed gastric motility, in mammals at least.

It is assumed that smooth muscle cells respond in a similar manner to exogenous stimuli *in vitro* as they do *in vivo*, with the exception of exogenous neural activity upon the cells, which is generally abolished upon excision. However, neural activity can be inferred by pharmacological neurotransmitter studies of smooth muscle cells, thus simulating neural neurotransmitter release or by field stimulation studies such as those conducted by Fänge and Grove (1979). Hence, correlates and assumptions can be made about humoral and neural factors that are pharmacologically applied to GI smooth muscle *in vitro*. As mentioned, slowed gastric emptying is accompanied by a slowing of

peristaltic wave propagation speed and frequency *in vivo*. Therefore, one would intuitively expect a slowed SPM in excised circular smooth muscle to be indicative of slowed peristaltic wave propagation *in vivo*. Similarly, increased contraction strength (PF) in response to exogenous stimuli could be representative of increased mechanical breakdown.

As mentioned, post-prandial PSp closure at the end of a propagating peristaltic wave is effected by humoral/neural stimuli and is related to nutritive value of the food item. This creates a barrier unable to be opened by peristaltically generated intraluminal pressure. The pyloric sphincter allows the passage of chyme, when lower levels of exogenous stimuli are acting upon it (Olsson and Holmgren, 2001). The relatively higher force of contraction of the stimulated pyloric sphincter has been shown to correlate with increased peristaltic wave conduction in the distal pylorus in humans (Duthie *et al.* 1971). Duthie *et al.* (1971) demonstrated pylorus peristaltic wave conduction to be four times faster than other regions of the stomach, where peristaltic wave propagation speed and frequency was slowed by nutritive meal ingestion. Therefore, one might expect an increased SPM in pyloric sphincter both *in vivo* and *in vitro*. Based on this evidence, it was assumed that increased PS contractility and SPM of spontaneous rhythmic contractions in the isolated tissue studies conducted in this thesis are indicative of slowed gastric motility patterns *in vivo*.

3.5.2 Serum Study

The significant sustained elevated tonus of circular smooth muscle upon application of the 1000- and 100-fold dilutions of the GDAS +ve serum to both the CS and PSp preparations strongly indicated the presence, or possibly a reduction in, a bio-active blood-borne factor or multiple factors at levels far greater or lesser than are present in fish unaffected by GDAS. This/these substance/s were able to modify the tonus of isolated healthy circular smooth muscle. The PS tissue was able to generate the greatest peak tension of 0.221 ± 0.037 $mg.mg^{-1}$ and 0.256 ± 0.083 $mg.mg^{-1}$ in 1000- and 100-fold dilutions respectively. CS tissue was less sensitive to this factor or factors, producing a peak tension of 0.073 ± 0.004 $mg.mg^{-1}$ and 0.116 ± 0.04 $mg.mg^{-1}$ in 1000- and 100-fold dilutions respectively. These changes in the amplitude of contraction was considerably less than maximal responses to CCK8 and gastrin-1, but the serum was greatly diluted. These data potentially provide evidence for the intestinal brake hypothesis in that they suggest that a change in the level or levels of a single or multiple circulating hormones/neurotransmitters produced in fish affected by GDAS act on gut musculature. Control fish were also presumably showing an intestinal brake response, as fish from both groups were fed on a continuous *ad lib* basis prior to sacrifice. Circulating hormones/neurotransmitters in these fish were presumably low enough that they were undetectable by the application of diluted serum to isolated circular smooth muscle *in vitro*. It is worth noting that serum addition to isolated smooth muscle resulted primarily in an increase in basal tonus while the GI peptides mostly stimulated spiking activity. Why this pattern of activity occurred remains unclear. It may be that multiple factors are combining synergistically in the blood to effect PSp closure in GDAS + fish.

As mentioned above, it is worth noting that blood-borne factors may have decreased in GDAS +ve fish. Particularly those factors associated with nutrient absorption (i.e. growth hormone (GH), insulin and insulin-like-growth factor-1 (IGF-1) and others), whose levels have been correlated with reduced nutrient absorption (Sigalet and Martin, 2000). This is anecdotally validated by NZKS reports of poor condition factor scores and reduced visceral fat stores in GDAS +ve fish (NZKS, pers. com). Again, GDAS may be a result of a synergistic effect of multiple humoral effectors occurring at levels not typical of healthy fish.

3.5.3.1 Spikes Per Min (SPM)

SPM in CS tissue challenged by CCK8 showed a negative linear relationship with increasing concentration. This *in vitro* response is suggestive of a slowing of peristaltic wave propagation, representing a slowing of gastric motility and mechanical breakdown of ingested food items *in vivo.* The SPM in PS challenged by CCK8 showed a positive hyperbolic relationship with concentration. A hyperbolic relationship could represent an attenuation of the response of pyloric stomach smooth muscle with high concentration of exogenously applied peptide. Despite being the best fit of the data (polynomial r^2=0.90, linear r^2=0.34), this relationship seemed unusual, considering linear regression fitted well to all the other treatments. Therefore, this observation warrants further investigation in order to confirm the hyperbolic relationship between concentration and SPM presented in the current study. PSp SPM for CCK8 showed a linear increase with concentration. The increased SPM in response to increased concentrations of CCK8 was interpreted as being representative of slowed gastric evacuation pattern *in vivo* which is thought to correspond with increased PF of contraction (discussed below). Since it is known that the rate of

wave conduction and contraction force increases in the distal pyloric region (sphincter) relative to more proximal regions in mammals during slowed gastric evacuation (Duthie *et al.* 1971), I extrapolated to the fish tissue used in the current study. Based on this extrapolation it can be assumed that an increased SPM in PS tissue *in vitro* might represent an increase in peristaltic wave conduction speed *in vivo*. No relationship was established between SPM and CCK8 concentration in the Int. This suggests that intestinal smooth muscle does not increase in terms of SPM in response to increased concentrations of CCK8, despite an increase in contractility in response to increasing concentration. The Int tissue data was quite variable (see error bars) and it is recommended that the relationship between SPM and concentration of CCK8 is revisited in future studies.

SPM in gastrin-1 challenged CS showed a similar response to that seen in CCK8; a significantly decreased SPM with concentration, which is also likely to represent a slowing of gastric motility and associated slowed mechanical breakdown of ingested food items *in vivo*, since slowed peristaltic wave propagation frequency has been shown in mammals exhibiting the ileal brake. Since pyloric stomach and pyloric sphincter tissues are continuous, the significant linear increase in PS SPM with concentration is also probably indicative of slowed gastric motility *in vivo*, via the same mechanism outlined for the pyloric sphincter in response to CCK8. As already outlined, the tissue is thought to respond in this way to assist in the prevention of digesta transit between compartments. The gastrin-1 PSp SPM data was remarkably similar to the values generated for CCK8 in terms of range and the positive linear relationship with increasing concentration observed. Therefore, a very similar effect is predicted *in vivo* in response to plasma CCKs and gastrins in the PSp. Unlike CCK8 challenged Int tissue, gastrin-1 challenged Int SPM

showed a slight positive, albeit non-significant, relationship with concentration. A slight positive relationship may be suggestive of increased motility. However, more replicates of the data are required to suggest anything other than that there was no real response generated in terms of SPM to increasing concentrations of gastrin-1. Therefore, similar to the response to CCK8, increased concentrations of gastrin-1 appear to cause increased PF of contraction but not SPM.

Contrary to the results for CCK8 and gastrin-1, 5-HT SPM in CS tissue showed a positive linear relationship with concentration. This is not indicative of slowed stomach motility *in vivo*. Instead this is suggestive of increased motility. However, similar to the responses to both CCK8 and gastrin-1, PSp SPM showed a positive linear relationship with increasing concentration of 5-HT. The concentration to elicit EC_{50} was significantly higher than those of the peptides. The high concentrations (low EC_{50}s) of 5-HT required to elicit EC_{50} in both CS and PSp may be typical of the kind of molecule it is, since neurotransmitters typically act in high concentrations at their active sites, although these are typically localised. Despite this, the pattern of activity in the PSp was consistent with the observations in the two peptides. Therefore, it may be concluded that 5-HT is likely to be involved in regulation of smooth muscle tonus in the PSp in Chinook salmon but is probably involved as a neural mediator, not a humoral factor.

3.5.3.2 PF and EC_{50} Values

Both CCK8 and gastrin-1 showed potent concentration-dependent effects on all the tissues examined (CS, PS, PSp and Int) in terms of PF and EC_{50}. TMS PF values generated were remarkably consistent in all tissues that received either CCK8 or gastrin-

1. CS tissue mean ± SEM PF generated for CCK8 (46.8 ± 13.2 $mg.mg^{-1}$) and gastrin-1 (36.3 ± 12.6 $mg.mg^{-1}$) were very similar. However, gastrin-1 was far more potent with a log10 EC_{50} ± 95% CI value of -12.45 ± 0.66 compared with an EC_{50} value of -8.15 ± 0.90 for CCK8. PS tissue TMS PF generated for CCK8 (32.2 ± 9.6 $mg.mg^{-1}$) and gastrin-1 (42.5 ± 1.0 $mg.mg^{-1}$) were also very similar with gastrin-1 eliciting a slightly greater PF. Gastrin-1 was also far more potent than CCK8 in the PS with a log10 EC_{50} value of -12.55 ± 0.63 compared with a log10 EC_{50} value of -7.88 ± 0.48 for CCK8. Int tissue displayed a similar trend with a comparable PF generated in response to CCK8 (69.2 ± 20.0 $mg.mg^{-1}$) and gastrin-1 (56.0 ± 10.9 $mg.mg^{-1}$). CCK8 produced a slightly bigger PF while gastrin-1 was more potent with a log10 EC_{50} value of -12.69 ± 1.12 compared with a value of -8.93 ± 0.64 for CCK8.

In a similar set of experiments in the Atlantic cod *Gadus morhua* gastric longitudinal smooth muscle strips were contracted by sulphated human CCK8 to a PF of 15.6 mN with an EC_{50} value of 7.42 ± 0.09 and showed increased amplitude of contractions (Jönsson, Holmgren and Holstein, 1987). Cerulein (amphibian CCK-like molecule) was the most potent CCK-like peptide to contract gastric smooth muscle with a EC_{50} value of 7.96 ± 0.12. However, the PF generated in response to cerulein was not as strong as that to CCK8, at 13.8 ± 1.3 mN. Unsulphated gastrin-17 in their experiments revealed a PF of 7.3 ± 1.5 mN with a EC_{50} value of 7.38 ± 0.10 while gastrin-5 produced a PF of 6.73 ± 0.11 mN with a EC_{50} value of 7.2 ± 0.9. In the experiments of Jönsson, Holmgren and Holstein (1987) they found that the forms of gastrin used were less potent than human sulphated CCK8, the same CCK used in the current study. Thus, Gastrin-1 was more potent than human sulphated CCK8, gastrin-17 and gastrin-5. These data are

consistent with the findings of the current study in that elevated amplitude and tonus of rhythmic activity were observed in the gastric smooth muscle of another teleost, the Chinook salmon. Furthermore, the EC_{50}s and PFs reported are similar to those in the current study.

Unsurprisingly, the most powerful tissue in terms of contractility was the PSp which generated a TMS PF of 155.2 ± 59.5 $mg.mg^{-1}$ for CCK8 and 162.0 ± 56.3 $mg.mg^{-1}$ for gastrin-1. In contrast to other tissues, gastrin-1 was not significantly more potent in its ability to stimulate force of contraction (log10 EC_{50} -9.35 ± 0.78), compared with CCK8 (log10 EC_{50} -8.98 ± 0.68). It may be significant that the two log10 EC_{50} values from gastrin-1 and CCK8 PSp tissue were the most similar in terms of potency. In all other tissues, gastrin-1 had far lower EC_{50} values when compared to CCK8 values within tissues, despite showing relatively similar PF values.

From these data it is evident that Chinook GI smooth muscle tissue is far more sensitive (log10 EC_{50} values) to gastrin-1 compared with CCK8 in CS, PS and Int tissue. This difference was approximately three orders of magnitude in most cases. However in the PSp tissue, CCK8 and gastrin-1 showed no difference in terms of log10 EC_{50}. While the CCK8 log10 EC_{50} for PSp smooth muscle was comparable (no statistical difference) to log10 EC_{50}s from elsewhere in the GI tract in response to CCK8, the PSp tissue TMS log10 EC_{50} value for gastrin-1 was approximately three orders of magnitude greater that the values from elsewhere in the GI tract. Therefore, GI smooth muscle cells show higher gastrin-1 sensitivity in all tissues except the PSp. The different sensitivity of PSp to gastrin-1 may suggest increased transport through the GI tract at low concentrations, with the intestinal brake being applied only as concentrations rise. This seems to fit well with a

model of normal intestinal brake function, in that the brake would be applied only when nutritive stimulus is sufficient to trigger changes in the PSp tonus.

The high sequence homology of CCK8 and gastrin-1 at the N-terminus and the similar EC_{50}s and PFs reported in the current study may suggest that both of these non-native peptides are acting upon the same receptor in the gut, with the gastrin-1 molecular structure more closely resembling a native CCK or gastrin. The native forms of gastrin in Chinook are probably similar to gastrins isolated from the teleosts, river puffer (*Tetraodon nigroviridis*) and Japanese flounder (*Paralichthys olivaraceus*) by Kurokawa *et al.* (2003). The native forms of CCK in the Chinook are probably similar to the CCKs identified in brown trout (*Salmo trutta*) by Jensen *et al.* (2001).

However, the different sensitivity of the PSp compared with other parts of the GI tract in response to gastrin-1 may suggest that a different, lower affinity, receptor for gastrin-1-like molecules may be present in the PSp tissue of Chinook. Therefore, it is more likely that CCKs and gastrins are acting in the Chinook as two distinct humoral systems. As mentioned, Jönsson, Holmgren and Holstein (1987) have shown gastrins and CCKs to have different effects on CS longitudinal smooth muscle contractility, with CCKs able to generate higher PF values (CCK8: 15.6 ± 1.9 mN net force) relative to a gastrin (gastrin-17: 7.3 ± 1.5 mN net force and gastrin-5: 7.2 ± 0.9 mN net force), as well as characterising gastrin/CCK endocrine cell distribution in the Atlantic cod through immunoreactivity studies. Interestingly, their work showed the greatest 'CCK/gastrin-like' immunoreactivity in the pyloric stomach, pyloric sphincter and the pyloric ceacae. In addition, earlier work by Holmgren *et al*. (1982) revealed that gastrin/CCK immunoreactivity was present in the pyloric region of the stomach and that gastrin-1 and

CCK8 circular and longitudinal smooth muscle contractility was increased in rainbow trout *in vitro.* Furthermore, these data are also consistent with the previously mentioned work of Kurokawa *et al.* (2003) who showed the presence of multiple gastrin and CCK genes in the puffer and flounder. All these data and the data presented in the current study suggest that both CCKs and gastrins are producing responses in teleost GI tissues that may be capable of producing a slowed gastric evacuation pattern. This is consistent, at least in the case of CCK8, with the findings of Olsson *et al*. (1999), who showed slowed gastric evacuation in brown trout, through intraperitoneal injection.

The GDAS +ve serum myography revealed that contractility of GI tissues was stimulated with very low concentrations (1000- and 100-fold dilutions) of serum. This would suggest that very high levels of circulating blood-borne factor/s are present in GDAS +ve fish plasma that may produce gastric evacuation patterns *in vivo* that are slowed beyond 'normal'. This observation is consistent with the results presented for gastrin-1, as both the serum and gastrin-1 operated at very low concentrations. GDAS +ve diluted serum application to PSp tissue produced greater PF values compared with CS tissue. This is consistent with the peptide/neurotransmitter myography and the smooth muscle integrity myography presented in Chapter 2. All these data suggests that PSp is able to generate higher TMS PF than CS.

Together with the SPM data, it appears that 5-HT is not a humoral factor acting in the GI tract. This conclusion was reached due to the high concentrations of 5-HT used in the current study and the minimal 5-HT smooth muscle contractility responses observed. The GDAS +ve serum dilutions suggested that minuscule concentrations of GDAS +ve serum were able to contract CS and PSp tissue. With a PSp log EC_{50} value of -6.18 ±

1.14 calculated for 5-HT it would seem unlikely that it was operating at concentrations much higher than these *in vivo*. However, it appears that 5-HT might be involved in neural stimulation of the GI tract. 5-HT produced much lower PF and much higher EC_{50} values for both CS and PSp tissues. The lower PF and higher EC_{50} values might suggest that 5-HT receptors are not as densely aggregated as those for CCKs and gastrins in Chinook GI tissue. This is not surprising considering the localised pattern of neural innervation in the GI tract of vertebrates, with neurotransmitter release (and associated receptors) located in specific areas (Matty, 1985). It is far more likely that CCK8- and/or gastrin-1-like molecules are effecting the 'over-stimulation' of the intestinal brake predicted by the intestinal brake hypothesis (Chapter 1).

The recently established role of GLP-1 in mammalian GI activity (Wôjdemann *et al.* 1999; Schirra and Göke, 2004; Brennan *et al.* 2005; Chelikani *et al.* 2005) was not demonstrated in the Chinook in the current study. This may be due to a number of reasons. The most likely reason is that the peptide used in the current study differs too greatly from the native peptide. Plisetskaya *et al.* (1986) isolated the native form of GLP from Coho salmon (*O. kisutch*), which comprised of 31 amino acids. Their work showed 12 amino acid substitutions in Coho compared with the aligned human GLP-1 (36 amino acid) sequence. Coho GLP differed significantly from human GLP-1 at the C-terminus, despite a mostly conserved N-terminal sequence. Recent work by H.C.G Prosser (2006) in our laboratory has highlighted the importance of using the native peptide, displaying variable C-terminus and conserved N-terminus sequences, in studies using human, rat and trout urotensin 2 on the rat and Chinook coronary arteries (pers. com.). The human GLP-1 used in the study may simply not have bound to a GLP-1-like receptor in the

Chinook GI tract, although Plisetskaya *et al.* (1986) did find weak Coho GLP radioimmunoassay cross-reactivity with human GLP-1. Alternatively, GLP may simply not be acting upon the GI tract of the Chinook. Further work on the effects of GLP in the Chinook GI tract is warranted. Despite the author being aware of the potential problems of using a non-native peptide, human GLP-1 was the most similar GLP to known teleostean GLPs commercially available at the time of going to press. Further work on GLPs in fish will be far more informative if native GLP are isolated and synthesised at the outset of the research.

In conclusion, it seems likely that GDAS +ve fish have a single or multiple bio-active blood-borne factor/s at levels significantly higher than GDAS –ve fish. This/these factor/s produced *in vitro* responses representative of the smooth muscle responses expected during an intestinal brake 'over-stimulation'. In addition, non-native CCK and gastrin contracted the pyloric sphincter muscle and modified SPM. These responses are capable of causing a slowed gastric evacuation pattern like that which occurs during the intestinal brake in teleosts (SPM, PF and EC_{50}s). Gastrin-1 displayed more of the characteristics of a hypothesised candidate mediator of the intestinal brake. The role of 5-HT and GLP in gastric evacuation in fish still remains ambiguous. Further work could focus on the isolation of the bio-active blood-borne factors identified in the serum study. Furthermore, native peptides and receptors should be purified and their sequences identified in order to elucidate humoral effects in any animal study.

CHAPTER 4

General Discussion

4.1 GDAS Epidemiology and Physiology

The data presented in this thesis suggest that GDAS incidence in farmed Chinook smolt is a consequence of the physical properties of the commercial feed pellets fed and the continuous drinking associated with a hyperosmotic environmental osmolarity. The FW and SW trials presented in Chapter 2 demonstrate this quite clearly. Two diets (A: low cohesion and B: high cohesion) were fed in order to test the effects of (1) osmotic environment and (2) physical properties of diet on GDAS development. From the data it is clear that the combination of a low cohesion diet and what was assumed to be copious and continuous drinking of the medium associated with living in SW results in GDAS manifestation in experimental populations. The histological data collected from these fish correlated with the morphological changes known to occur in GDAS affected fish in commercial cages (NZKS; internal report). Thus the experimentally induced fish used for data collection in the current study were deemed to be in a similar physiological state as GDAS affected populations in commercial operations. Therefore, experimentally induced fish in the current study were used to characterise the progressive changes in physiology and morphology of GDAS affected fish. Hence, correlates between historical outbreaks of GDAS and GDAS affected fish in the current study can be made.

With these correlates established, it appears that GDAS development in commercial operations involves a loss of stomach contractility, while pyloric sphincter contractility increases. This suggests that physiological processes are occurring in

animals displaying GDAS symptoms in response to inappropriate pelleted feed in SW. These processes result in quite different responses of GI smooth muscle, in adjacent tissues of the stomach, in response to cholinergic stimulation. Similarly, smooth muscle cell depolarisation with KCl revealed that fundamental changes to GI smooth muscle contractility can occur over a short period of time.

In addition, osmoregulatory homeostasis regulated by the pyloric caecae was found to be impaired in GDAS developing fish in the caecal fluid transport experiments. These data corresponded with the increased serum osmolality (dehydration) of SW acclimating smolt fed diet A, relative to non-GDAS developing fish fed diet B. Dehydration may exacerbate GDAS in itself, as the thirst response will be highly active in fish with plasma osmolality elevated above 'normal'. Even if increased drinking occurs in those fish, homeostasis appears not to be maintained. Further work is warranted into the changes that occur in osmoregulation as GDAS develops, especially the mechanisms that inhibit function of the pyloric caecae.

As mentioned earlier, causal factors in GDAS have been identified. These factors are thought to combine in the fish to result in intestinal brake dysfunction, explained here by the 'intestinal brake hypothesis' (Chapter 1). Data collected thus far on GDAS development seem to fit with this proposed mechanism. However, the possibility still remains that the mechanism proposed here is in fact not the only mechanism of GDAS development. The presence of some pharmacological agent, such as high levels of tryptophan in the feed, might also contribute to GDAS. In his review of the relative potencies of amino acids to stimulate delayed gastric emptying, Heading (1980) found that tryptophan was by far the most potent. However, the lack of GDAS in FW fish fed

diet A indicate that a possible toxic effect is insufficient to trigger GDAS. Instead it seems that diet cohesion and the rapid disaggregation of the fine particulate feed pellets fed combine to cause GI dysfunction in SW fish. Therefore, based on the data, the author accepts the intestinal brake hypothesis as the mechanism for GDAS development.

4.1.1 Diet Cohesion

The two diet comparison (Chapter 2) conducted in the current study showed that diet A rapidly disaggregated in both the stomach and in the NZKS pellet cohesion test, while diet B did not. The low cohesion diet, diet A, has also previously been associated with high GDAS incidence in their commercial operations. By feeding diet A under controlled conditions, we were able to gauge both the time of onset and the percentage of the population affected by GDAS over time. It was found that GDAS symptoms were present (12.5%) in the population at two weeks post-SW transfer on feeding diet A. Incidence increased weekly, reaching 87.3 % by week 5. The feeding of this diet in FW and the feeding of diet B in both FW and SW did not produce GDAS. Thus osmotic environment and diet cohesion were shown to be correlated with GDAS incidence, although either factor alone was not sufficient to trigger it.

The primary determinant on whether a fish in SW will develop GDAS appears then to be the physical cohesion of the diet it is being fed. Cohesion of the diet is thought to be inter-related with particle size, which is discussed further below. These two physical factors are thought to exert deleterious effects on the GI tract through the mechanisms of the intestinal brake hypothesis, due to the constant supply (and consumption) of commercial feed in most salmon farming operations (NZKS, pers. com.). As mentioned earlier, this continual high nutrient feeding is unlike anything encountered naturally by

Chinook. This in itself may place stress on the GI system. The role of 'causal' and exacerbating stressors are discussed further below in relation to GDAS development.

4.1.2 Feed Pellet Particle Size and Lipid Content

The properties of the food consumed during feeding have a profound effect on the nature of gut motility in vertebrates (dos Santos and Jobling, 1988). Jobling (1987) has shown that particle size and dietary energy content, particularly high lipid content, affects gastric evacuation in fish by presenting the absorptive epithelia of the intestine with an 'overload' of nutrients. It is thought that although particle size and lipid content is a vitally important premise of the intestinal brake hypothesis of GDAS development, the rate of delivery of these fine particles and lipids to nutritive receptors in the pyloric caecae/intestine is what is critical in determining motility patterns.

Since manufacturing processes for aquaculture feeds are well established and many of the ingredients are already fine ground when received (B. Wybourne, pers. com.), it would be quite difficult to stipulate specific 'large particle size' pellets. This may also interfere with the digestibility and the digestible energy content of the pellets (Halver, 1989). Although large particle size may be a beneficial characteristic of feed pellets in preventing GDAS, feed conversion ratio (FCR) could potentially be expected to increase, as less of the ingested feed might be able to be utilised by the animal. Since most commercial operations rely on fast growth rates to ensure profitability, this type of action could jeopardise economic viability of a company. Consequently, based on this knowledge and the data presented on gastric evacuation patterns in this thesis, modifying physical cohesion of fine particle ingredients is the most feasible and direct way to mitigate the problem of GDAS. Prevention and mitigation are discussed further in 4.3.

4.2 Exacerbating factors in GDAS

4.2.1 Stress

The physiological stress response is an adaptive mechanism to real or perceived challenges to an organism's ability to meet its real or perceived needs (Greenberg *et al.* 2002). Stress, as defined here, is a condition in which the dynamic equilibrium of an organism, or homeostasis, is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors (Wendelaar Bonga, 1997). These stressors have the potential to induce a stress response in an organism. The onset of the stress response is dependent on the organism's ability to resist these intrinsic or extrinsic stimuli (Bradshaw, 2003). Stressors can take the form of biological (e.g. competition, parasitism, pathological, reproductive), physical (handling, culture methods, experimentation), environmental (temperature, salinity, dehydration, chemical) and psychological stressors (confinement, overcrowding) (Greenberg *et al*. 2002; Wendelaar Bonga, 1997; Bradshaw, 2003). While many stressors can evoke dramatic neural and endocrine responses, a more modest or 'subclinical' response may be exhibited in response to milder stimuli (Greenberg *et al*. 2002). The physiological consequences of short-term, high intensity stress vary significantly from long-term low intensity stress. A good example of a situation where this is likely to occur is in fish aquaculture operations. Fish in aquaculture operations face challenges to physiological systems as a result of culture procedures, in addition to those occurring naturally (Wedemeyer, 1990).

Stress has been the focus of physiological studies for many decades and has been directed at a diverse range of phylogenetic groups in many different environments (Wedemeyer, 1996). Hans Seyle, a famous clinician, was a pioneer in the field of modern

stress research and wrote several classical papers on the subject. A paper published in Nature (Seyle, 1936) identified what he called a 'general stress response'. In this paper he clearly identified what he termed the 'stress triad' of adrenocortical enlargement, atrophy of the thymus and ulceration of the digestive tract, observed in experimental animals subjected to a wide variety of nociceptive (i.e. harmful) agents (Bradshaw, 2003). In his 1946 paper entitled 'The general adaptation syndrome and the diseases of adaptation' he communicated his now famous 'general adaptive syndrome' theory, comprising three stages: (1) a stage of 'alarm', (2) a stage of 'adaptation' and (3) a stage of 'exhaustion' (Seyle, 1946). Observations made on his patients led him to hypothesize that a general stress response was elicited by a wide range of stressors.

The stages of the stress response proposed by Seyle, have been renamed and adapted in subsequent characterisation of the stress response in vertebrates by other authors, but most are based on Seyle's general adaptive syndrome. One such classification is that given by Iwama *et al*. (1999) for the fish stress response. It identifies a primary response which involves the perception of an altered state and initiates a neuroendocrine/endocrine response that forms part of the generalised stress response in fish. Primary control of the stress response in teleost fish is through neural stimulation of the endocrine organs, which in turn secrete endocrine factors that are transported via the cardiovascular system to the target tissues/cells (Iwama *et al*. 1999). The secondary response involves the various biochemical and physiological adjustments associated with stress and is mediated to some extent by the stress hormones (Iwama *et al*. 1999). The tertiary response represents whole animal and population level changes associated with stress (Barton and Iwama, 1991; Iwama *et al*. 1999). If stress acclimation is unattainable

by the fish, substantial changes to the physiology of the whole animal typically occur. Energy repartitioning in the form of substrate delivery results in a shift away from anabolic activities such as reproduction and growth (Barton and Iwama, 1991). Long-term effects, dependent on duration and intensity, can lead to a decrease in growth, pathological resistance (through a suppressed immune system), reproductive success and swimming performance (Barton and Iwama, 1991). Consideration of stress induced changes in physiology is vital when considering any health related issue in aquaculture.

4.2.2 Stress on Stress

GDAS development in Chinook has been shown in this thesis to be a result of multiple factors. These factors can be regarded as stressors, based on the definition used here. These stressors are dietary (physical properties of artificial feed pellets) and osmoregulatory (active homeostasis in hyperosmotic media). The dietary stress is that placed upon the GI system by the rapid breakdown of the artificial fine particulate low cohesion pelleted feed. The resultant artificially high nutrient load placed upon on the intestine and stomach by the rapid disaggregation of these pellets is unlike any natural food item likely to be encountered by the animal in its natural environment (Higgs, 1995). The osmoregulatory stress is the stress of abrupt SW transfer and the associated energetic cost of actively maintaining plasma osmolality in this medium. Higgs (1995) reviewed the natural life-history of Chinook in its native rivers. He outlined how the ‘ocean type’ Chinook will remain in brackish water, mostly in estuaries, for a variable period. The gradual change from hyposmotic (FW river) to strongly hyperosmotic (SW) environments mitigates osmoregulatory stress. This drastically contrasts with fish in

commercial operations which are not afforded this acclimation period. Consideration of life-history is vital if real stress reduction is to be achieved in aquacultured fish.

It seems intuitively obvious that the two primary stressors identified in the current study, combined with the multiple stressors associated with fish aquaculture might accumulate to produce chronically stressed fish populations. If classified by the system of Iwama *et al*. (1999), GDAS developing fish would best be described by the tertiary response. This idea is backed up by anecdotal accounts of increased GDAS incidence in the presence of high levels of stress, in NZKS operations (NZKS pers. com.). For example, temperature in excess of the optimal range for Chinook has been shown to be a co-factor in GDAS incidence, as well as a potent stressor in itself (NZKS pers. com.). Unusually high temperatures, by Chinook standards (>14˚C; Healey, 1991), may result in exacerbation of GDAS development by mechanisms predicted in the intestinal brake hypothesis, by increasing metabolic rate via the Q_{10} effect. Elevated metabolic rate is known to (1) stimulate increased eating and drinking behaviour, (2) enhance metabolic processes via mass action and (3) increase swimming behaviour, thus increasing interactions with other fish (Wedemeyer, 1996). Similarly, other stressors may exacerbate the development and persistence of GDAS in Chinook salmon.

It is worth noting that upon initiation of the SW trial an unintentional and unexpected increase in dissolved nitrogenous wastes, above ‘safe operating levels’, occurred due to the holding tank biofilter being overloaded beyond its detoxification capacity. Several water changes and a biofilter upgrade reduced waste nitrogen to below ‘safe levels’ relatively quickly (13 days). Nitrite/ammonia toxicity was evident in some fish over this period, with a small number of nitrite associated mortalities resulting. The

increased levels of stress encountered due to poor water quality are thought to have acted as a co-factor in GDAS development in the current study by increasing stress levels in the fish. Anecdotal accounts from earlier attempts to experimentally induce GDAS suggested that high incidence was often correlated with the highest levels of stress (i.e. high temperature and nitrite/ammonia levels) (NZKS; pers. com.).

4.3 GDAS Prevention and Mitigation: Answers for Industry

Prevention: physical cohesion properties of feed pellets used in commercial operations and the use of 'natural food' diets

By examining the relationship between diet cohesion and GDAS in this thesis, it is clear that rigorous adherence to manufacture processes that produce a pelleted feed that reduces the rate of disaggregation in the stomach is necessary for GDAS prevention in commercial operations. Manufacture processes in aquaculture feeds are outside the scope of this thesis and will not be discussed in detail. However, the end product of these processes should result in a 'slow release' pellet that will deliver nutrients to the intestine at a rate that does not exceed the intestinal epithelial absorptive capacity. Such a product can be tested *in vitro* by a number of methods, providing that they replicate the processes known to occur in the stomach during digestion. Simple wetting, spinning, sieving and subsequent weight analysis of pellet samples can fulfil these criteria satisfactorily. If this screening test can be done reliably, before a given batch of feed is fed, then potentially harmful batches can be avoided. This has recently been made common practice in NZKS operations, with great success.

Communicating necessary information to feed manufacturers is vital if GDAS is to be prevented. However, despite compliance, inter-batch variation in terms of ingredients process and packaging are unavoidable and should be strictly monitored so that they fall within acceptable limits. These limits need to be set by the industry. Compliance with consumer demands is difficult to assure, therefore the author recommends an industry initiated 'double check' with their own tests of pellet cohesion properties.

Replacing the commercial feed pellet system used on almost all salmon farms with a 'natural diet', might altogether banish GDAS to the literature. A natural diet might consist of live or dead whole fish, invertebrates etc. This should intuitively not stress the GI system to the degree that artificial pellets have been shown to (Jobling, 1986; current study). In most cases, the feasibility of feed delivery in commercial operations excludes this option. In addition, 'natural feed' cost may make commercial operations less profitable, as well as increasing the risk of disease. Furthermore, growth may be slowed by the patchy nature of live 'natural feed' in commercial operations and the relatively lower nutrient content of this type of feed. Therefore, the use of 'natural feed' is thought to be unrealistic in commercial operations.

As discussed, osmoregulatory stress as a result of abrupt transfer to SW has been shown to be a causative factor in GDAS. Therefore, a gradual acclimation to brackish water before SW transfer might mitigate the osmoregulatory stress currently experienced by fish in commercial operations. This would better replicate the natural life-history of the animal (Higgs, 1995). During this period, withholding feed or the feeding of a maintenance diet may reduce nutritive load on the GI system. A withholding period to allow physiological GI changes to occur post-transfer may also reduce stress during

vulnerable periods. This is not currently done in NZ operations (NZKS, pers. com.). Instead, due to logistical and financial reasons smolt are abruptly transferred to SW and immediately put onto a maximal feeding regimen.

Mitigation: suitable feed availability and maintenance rations. Can GDAS be reversed?

Once a given farm population shows high GDAS incidence, mitigation and reversal of the situation is highly desirable. Stock investment recovery is the absolute minimum acceptable for most commercial operations. Large consistent shortfalls in supply of quality fish, due to affected stock, could easily bankrupt a business in a number of years. Since it is currently difficult to maintain profitability of farming salmon in New Zealand, due to readily available cheap salmon products from both emerging (e.g. Chile) and established (e.g. Norway) salmon farming countries (NZKS, pers com.), any large scale pathological outbreaks may seriously threaten the financial viability of NZ operations. This would not only be detrimental for staff, shareholders and regional economies, but the country as a whole, since salmon products account for the second largest aquaculture export in dollar terms (c.$41 Million), after the greenlip mussel (*Perna canaliculus*) (SeaFIC, 2005).

As predicted by the intestinal brake hypothesis (Chapter 1), continued intestinal nutrient overload due to satiation feeding of high nutrient (low cohesion) feed pellets in SW will result in GDAS. Therefore, should a population show early signs of GDAS (e.g. abdominal distension), reduced nutrient load on the intestine should be effected by the modification of feed source and frequency of feeding. The feed that is causing/has caused GDAS should be discontinued from use and substituted with a high cohesion feed. It is

the opinion of the author that GDAS developing and affected fish populations should be fed maintenance diets in an attempt to halt the pathological progression of GDAS. Intrinsic stressors such as those predicted to occur by the intestinal brake hypothesis should be abolished by this action.

It may be that a fish subjected to this 'treatment' will not recover from GDAS, particularly if the fish is chronically affected. Anecdotal reports have revealed that simple diet change will not reverse the syndrome (NZKS, pers. com.). However, feeding frequency was not reduced in these 'trials'. Intestinal nutrient load reduction may have been insufficient to 'reverse' GDAS or it may simply be that the affected tissues may be irreversibly altered in terms of morphology (e.g. stomach smooth muscle atrophy). If affected tissues are able to recover, causative factor removal could result in a 'cure' for GDAS affected fish. This hypothesis remains to be proven. However, if appropriate preventative measures are taken before the feeding of stocks, GDAS should not develop in a population in the first instance.

4.4 Further Work

There are innumerable further experiments in a number of physiological and aquaculture research areas that could be designed from the work conducted in this thesis. Only the experiments deemed to be most informative by the author will be discussed. Causative factors in GDAS are now thought to be identified and some of the mechanisms of GDAS physiology have been characterised. However, GDAS physiology appears to be a complex summed reaction by the fish to multiple acute stressors. The most informative studies in elucidating further mechanisms of GDAS development and persistence could

be: (1) further elucidation of the relationship between changes in smooth muscle morphology and contractility, particularly the opposing responses in the CS and the PSp. (2) The slowed caecal water uptake rates in GDAS +ve fish reported in the current study could be correlated with physiological and biochemical changes in the animal, since the mechanism resulting in the relatively lower rate of fluid movement in isolated caecae still remains unknown. (3) Investigation into the cellular changes in the fluid-exposed epithelia in both the intestine and the gill could reveal further mechanisms in GDAS development. This could possibly be investigated by histological methods. (4) Further to this, drinking rates in GDAS +ve fish relative to GDAS –ve fish should be quantified in order to confirm the assumed constant and copious drinking predicted by the intestinal brake hypothesis. In the current study, the pattern and rate of gastric evacuation has been evaluated in healthy fish fed two diets with different cohesion properties. (5) Elucidation of the pattern and rate of gastric evacuation in GDAS +ve fish would be extremely informative in the understanding of the syndrome. This could be done by the same method used in the current study (serial slaughter) or by the use of inert markers and/or radiology studies. This was not attempted in the current study due to a lack of affordable equipment and the availability of usable GDAS +ve fish. Blood borne factor/s potentially capable of producing an overactive intestinal brake response have been identified in GDAS +ve serum in the current study. Pharmacological investigations into what those potential blood-borne factors are pointed towards gastrin-like and/or CCK-like molecules. (6) Assay of serum from GDAS +ve fish for either of these two peptides with reference to healthy fish levels would be highly informative.

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Appendix

Detailed Histology Methods

Preparation of Sections

Tissue was fixed and stored in 10% phosphate buffered formalin. The formalin was 'washed out' of the tissue with several changes of distilled water, about 1-hour each for a total of 3 hours. The tissue was then lightly stained in Mayer's double strength haemalum (Sigma), in order to easily orient the tissue in the wax block. The stain was then 'blued' in running tap water (mildly alkaline) for 10 minutes. The tissue was then dehydrated in an ethanol series, remaining in the each solution for half an hour; 50%, 70%, 90% (in distilled water) and then finally into absolute ethanol for 3 washes $1^1/_2$ hours total. After dehydration, the tissue was placed in Histoclear (Sigma) for 3 hours, with agitation until translucent. The tissue was then infiltrated with Ralwax under vacuum for a total of 3 hours by placing the tissue in a ceramic crucible filled with liquid Ralwax (at 60°C) the crucible was then placed in a vacuum chamber, running off a tap. Once fully infiltrated, the tissue was set in a Ralwax block and allowed to harden overnight. Once hardened, the block was trimmed to size and mounted on a wooden block and fixed in place with wax. The block was then mounted into a Leica® microtome where it was aligned. Five μm and 7 μm section ribbons were then cut. The ribbons were trimmed to size and placed on a cleaned glass slide smeared with slide adhesive. The slides were then flooded with distilled water to 'float' and decompress the ribbon by heating the slide and water on a heat pad. The slides were then drained of excess water and allowed to dry completely in an oven at 37°C for 24 hours.

The slides were then stained as follows:

1. De-waxed slides in warm xylene (40-50°C) for 5minutes
2. Rinsed in 50/50 xylene/absolute ethanol (3dips)
3. Absolute ethanol for 2 min
4. 70% ethanol for 2 min
5. 70% ethanol for 2 min
6. 50% ethanol for 2 min
7. Distilled water for 2 min
8. Double strength Mayer's haemalum (without acetic acid) for 4 min
9. Blued the stain in running tap water (slightly lkaline) for 10 min.
10. 50% ethanol for 2 min
11. 70% ethanol for 2 min
12. 70% ethanol for 2 min
13. Absolute ethanol for 2 min
14. Counter-stained in 0.5% Eosin Y (Sigma) in absolute ethanol for 5 min.
15. Absolute ethanol for 1 min, to differentiate the EosinY .
16. Rinsed in 50/50 xylene/absolute ethanol (3dips)
17. Sections cleared in xylene (at 20°C) for 3+ mins
18. The slides were then mounted in Eukitt and a 60mm coverslip and allowed to dry for 48 hours in a 37°C oven.

Slides of a representative sample of fish fed either diet A or diet B were prepared for each week of both the FW and SW trials.

Examination of Sections

Sections were examined by a Zeiss Microscope (Model AXIO-Imager M1) with an inbuilt camera taking pictures at 5x magnification. Measurement were made from the images taken of the slides. Each week/diet treatment was represented by three slides prepared from the stomach tissue of three individuals (i.e 9 slides for each treatment and n=6 for each weekly comparison). The samples were randomly chosen from the group of fish killed weekly for other experiments. Measurements were made of inter-rugal fold distance and circular smooth muscle width at the middle of a rugal fold (Figure 2.21).

Five measurements, from different sections were taken from each slide and averaged to give a single value for each individual.