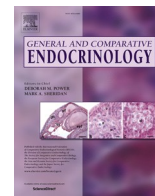




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Research paper

## Cholecystokinin and gastrin-releasing peptide differentially inhibit appetite of rainbow trout

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## ABSTRACT

The appetite in fish is a multifaceted phenomenon that comprises specialized interactions between brain and peripheral signals, and as a result, appetite is either stimulated or inhibited. Cholecystokinin (Cck) and gastrin-releasing peptide (Grp) are two postprandially released gastrointestinal peptide hormones that affect feed intake in fish. As the stimulatory or inhibitory effects of hormones can vary in duration and strength, making the nature of hormone effects dynamic, we modelled the dynamics of Cck and Grp using a direct, non-stressful approach. Fish were hormonally treated through an intraperitoneal cannula and feed intake was monitored for 12 h post-injection using a self-feeder system. Cck and Grp decreased feed intake in a dose-dependent manner, hormone-specific both in terms of magnitude and duration. Cck had an immediate inhibitory effect on feed intake, which lasted two–three hours, whereas the immediate inhibitory effect of Grp lasted for the entire 12-hour observation period. The data suggest that Cck acts as a short-term satiety signal in rainbow trout, while Grp acts as a longer-term appetite suppressor.

## 1. Introduction

Appetite in fish is centrally regulated, primarily by areas of the hypothalamus, which receive and process information from hormonal, metabolic and neural signals. The centrally integrated assessment of the nutritional and energetic state of the fish, results in the desire to feed (appetite) or not (satiety) (Rønnestad et al. 2017; Volkoff, 2016). The gastrointestinal tract plays a key role in this process, as it produces several peptides, some of which are released in response to nutrients and/or fullness in the gut and having functions important for digestion of the feed as well as influencing appetite and meal size. Cholecystokinin (Cck) and gastrin-releasing peptide (Grp) are two gut peptides related to post-prandial activity of the gastrointestinal tract (Aldman et al. 1992; Guilloteau et al. 2006; Olsson et al. 1999; Volkoff, 2016; Holmgren and Jonsson, 1988; Holstein and Humphrey, 1980; Thorndyke et al. 1990). Cck is mainly produced in the intestine in most fish groups (Jönsson and Olsson, 2024) and Grp, homologous to bombesin, is produced and secreted by enteric neurons in the gastrointestinal tract (Dockray et al. 1979; Thorndyke et al. 1990; Vigna and Thorndyke, 1989). Even though there are studies addressing the role of Cck and Grp on feed intake, the

strength and duration of their effects have received less attention and there are contradictory data on their effect among species and studies.

Studies with different approaches support the role of Cck as a hormone that increases satiety in the short term in several fish species. Thus, Cck injections inhibit appetite in goldfish, *Carassius auratus* and largemouth seabass, *Micropterus salmoides* (Himick and Peter, 1994a; Liang et al. 2024), as well as Siberian sturgeon, *Acipenser baerii* (Zhang et al. 2017). Similarly, oral Cck-capsule administration inhibits appetite in European seabass, *Dicentrarchus labrax* (Rubio et al. 2008). Further, treatment with Cck receptor antagonist increases appetite in coho salmon, *Oncorhynchus kisutch* (Löhmus et al. 2008) and rainbow trout, *O. mykiss* (Gélineau and Boujard, 2001). Plasma Cck levels increase after feeding in rainbow trout (Jönsson et al. 2006) and Cck expression levels increase following a meal in pyloric caeca of yellowtail, *Seriola quinqueradiata* (Murashita et al. 2006), further supporting the role of Cck as a satiety factor. However, some contradicting data exist. Thus, Cck gene expression in the gut increases during winter fasting of coho salmon (Löhmus et al. 2008) and peripheral Cck injections did not affect feed intake in channel catfish, *Ictalurus punctatus* (Schroeter et al. 2015).

The role of Grp in feed intake is more unclear. Intraperitoneally

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injected bombesin suppress feeding behavior in goldfish, *C. auratus* (Himick and Peter, 1994b), and Grp mRNA levels in the gut decreased during fasting in zebrafish, *Danio rerio* (Koven and Schulte, 2012), in line with an appetite-inhibitory action. However, feeding or diet composition does not affect plasma Grp levels in rainbow trout (Jönsson et al. 2006) and only daily ration size, but not feeding *per se*, affected Grp expression in the gut of Atlantic cod, *Gadus morhua*, and further no change was measured after long-term fasting or refeeding (Xu and Volkoff, 2009). Thus, while some studies have indicated that Grp has an anorexigenic action in fish, other studies contradict this.

Dominance and stress are known to affect fish appetite (Best et al. 2023; Conde-Sieira et al. 2018), which may cause disturbances in the study. To minimize the impact of external factors in experiments, fish could be held in separate and as undisturbed conditions as possible. In addition, appetite and feed intake are not directly comparable, as appetite may arise without the possibility of feed intake. However, self-feeder systems which allow the fish to obtain feed whenever they desire to eat create a laboratory setting where changes in their voluntary feed intake can justifiably be interpreted as changes in appetite.

The aim of this study was to elucidate the quantitative and temporal effects of Cck and Grp on the appetite of rainbow trout. To do so, a relatively stress-free *in vivo* approach in a computerized self-feeding system was used. Individually housed and pre-adapted fish were treated with different doses of Cck and Grp, administered through intraperitoneal cannula, and voluntary feed intake monitored over a 12-hour period. The obtained data allowed the modeling of how Cck and Grp affect the short-term appetite of rainbow trout.

## 2. Materials and methods

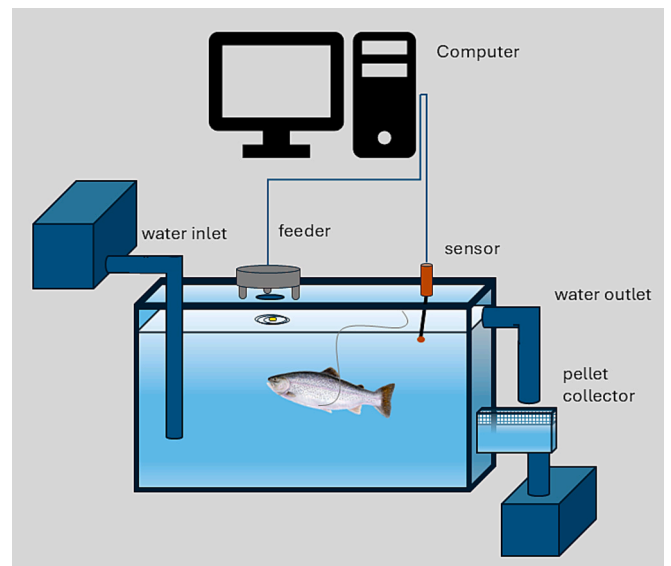
### 2.1. The fish, acclimation and operation

The trials were conducted on sexually immature rainbow trout, raised at a land-based aquaculture facility at the Finnish Game and Fisheries Research Institute (now Natural Resources Institute Finland) in Tervo.

The fish were transported to the Finnish Game and Fisheries Research Institute experimental facility in Evo, kept in holding tanks and given minimum two weeks to recover from the transport. Then, the fish were placed in the individual, experimental computer-controlled self-feeder tanks (Fig. 1), modified from Boujard et al. (1992) for a four-week acclimation period, during which fish learnt how to use the self-feeder system. Throughout the study, the fish received amino acid balanced commercial dry feed for rainbow trout with 30 % lipid content (Raisio Ltd, Finland). Fish used in the experiments had an average weight of 943 g.

The fish were cannulated under MS-222 anesthesia (100 ppm, buffered with sodium bicarbonate) to allow for a later, handling-free intraperitoneal hormone administration. The cannula (polyethylene, 60 cm, od 1 mm) was inserted through an incision in the abdomen with the tip positioned in the peritoneal cavity near the pylorus. The cannula was fastened under the skin of fish with a thicker polyethylene tube through which the cannula passed. The cannula was filled with saline and the fish were returned to their tank for a 96-hour recovery before the experimental protocol was initiated. Studies were carried out in accordance with *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* and with *Finnish Animal Welfare Act*. An animal experimentation permit was approved for the experiment, and the surgical procedures were conducted by personnel qualified to conduct animal experiments.

For each experimental series, twenty fish were housed individually in 120-liter tanks (Fig. 1). The tanks were supplied with partially recirculating freshwater at 5 l min<sup>-1</sup> that passed a QL-240 UV-sterilizer, and a temperature controller keeping the water at 12 to 14 °C. The tanks were fully covered to avoid external disturbance, and equipped with their own light source, keeping a 12:12LD photoperiod regime.



**Fig. 1.** The computer-controlled self-feeder system. It consists of an inductive sensor, a computer and a feeder. When the fish pushes the rod attached to the sensor, a signal is sent to the computer as a request. The request is stored on the computer and at the same time, the computer starts the feeder, which drops one floating feed capsule on the water surface. To separate requests, the computer only accepts a second request after the feeder has delivered the first capsule and stopped. If the capsule is not consumed, it will float out of the tank and into a collector. When the fish is treated, the tank lid is lifted and the floating catheter is grabbed with minimal disturbance to the fish, the injection carried out and the catheter and lid returned.

The self-feeders (Fig. 1) were operational only during the 12 h light period. By pushing the vertical sensor-rod of the self-feeder, the fish would request feed. Each push was registered as a feed request to the computer and one gelatin capsule containing feed would fall into the tank. Each capsule (435 mg) consisted of 95 mg gelatine capsule and 340 mg dry feed. No extra ingredients were added. The feed was encapsulated in gelatin capsules to make it fully buoyant. If the fish did not eat the capsule, it floated out of the tank to a collector. Any uneaten feed was removed from the hourly feed intake data.

### 2.2. Hormones and injection doses

The Cck used for treatment was a rainbow trout Cck-8-S with the sequence Asp-Tyr(SO<sub>3</sub>H)-Leu-Gly-Trp-Met-Asp-Phe (Johnsen 1998), synthesized by Asynth Service BV, Netherlands. Cck-8-S was used as the mammalian counterpart has been shown to have physiological effects in rainbow trout (Olsson et al. 1999), differing only to the trout form by having methionine and not leucine in the sixth position from the c-terminal. The rainbow trout Grp used was synthesized by MedProbe A.S., Norway based on the sequence Ser-Glu-Asn-Thr-Gly-Ala-Ile-Gly-Lys-Val<sup>10</sup>-Phe-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly<sup>20</sup>-His-Leu-Met-NH<sub>2</sub> obtained by Jensen and Conlon (1992). For modeling purposes, a wide

**Table 1**

The doses of cholecystokinin (CCK) and gastrin releasing peptide (GRP) administered to the fish through intraperitoneal cannula during the experiments (see Table 2 for treatment schedule).

Dose	Abbreviation
0 nmol kg <sup>-1</sup> fish	D0
0.05 nmol kg <sup>-1</sup> fish	D0.05
0.5 nmol kg <sup>-1</sup> fish	D0.5
2.5 nmol kg <sup>-1</sup> fish	D2.5
5.0 nmol kg <sup>-1</sup> fish	D5

range of peptide doses were given, from 0 to 5 nmol kg<sup>-1</sup> fish (D0 to D5, Table 1). The doses were delivered in an injection volume of 50 µl kg<sup>-1</sup> fish, through the intraperitoneal cannula, followed by the flushing of the cannula by 0.1 ml of saline.

### 2.3. Experimental design

Two separate experiments were carried out to test the effects of Cck and Grp on voluntary feed intake of rainbow trout. The experiments were identical in design; each was carried out over 10 days, consisting of two 3-day injection periods separated by a 4-day resting period (Table 2). Modified Latin square design was utilized, where the fish received two different hormone doses and D0 on consecutive days. This design allowed an estimation and adjustments for the sequential nature of the trial so that the effects of the day and the order of dosing could be modelled and adjusted for. As the fish were randomly sub-divided into three treatment groups (Table 2), each hormone dose was administered each day. During the first treatment period, the doses given were D0, D0.05 and D5, whereas during the second period, the doses given were D0, D0.5 and D2.5. The daily injections were administered to the freely swimming fish via the intraperitoneal cannula without any observable disturbance and given in the morning as soon as the lights in the tanks were turned on. The voluntary feed intake of the fish was monitored for 12 h after each injection.

The function of the cannulas was tested after every 3-day injection period. Fish with non-functional cannula, due to connective tissue growth, were excluded from the studies. The numbers of fish included in the data analysis are shown in Table 2. All fish were sacrificed at the end of the experiments.

### 2.4. Statistics

Both peptide experiments were modelled separately, but in identical manner. In both statistical models, the response was the cumulative number of capsules eaten each hour after the injection. Because the capsule response is a discrete count, the negative binomial family of distribution was assumed in the modeling. This accounted for the possible overdispersion in the capsule counts and the discrete nature of the response. The predictors in the model were the time (hours after injection), the peptide dose injected, and the previous peptide dose

**Table 2**

Hormonal treatment protocol for the two 10-day experiments, testing the effects of CCK and GRP, respectively. On day 1, 20 fish were randomly divided into three treatment groups, and injected for three days (days 1–3) with a daily hormonal dose as shown. Then, the fish were “rested” for four days (days 4–7). On day 8, the fish were again randomly divided into three treatment groups and injected for three days (days 8–10) with a daily hormonal dose as shown. The functionality of the cannula was tested on day 3 and 10. Fish with non-functional cannula, due to connective tissue growth, were excluded from the studies. The number of fish included in the data analysis are shown.

Days	Treatment group	Injection scheme (dose and order)			Number of fish	
		Day 1	Day 2	Day 3	CCK	GRP
Days 1–3	Group 1	D5	D0	D0.05	5	7
	Group 2	D0.05	D5	D0	6	6
	Group 3	D0	D0.05	D5	6	6
Days 4–7	Resting period					
Days 8–10	Group 1	Day 8: D2.5	Day 9: D0	Day 10: D0.5	6	6
	Group 2	D0.5	D2.5	D0	5	7
	Group 3	D0	D0.5	D2.5	6	5

injected. The latter was to adjust for the differences in the order the different doses were applied. The doses were treated as discrete predictors while the effect of time was modelled with a penalized spline to avoid assuming any prior form for the time effect, but let the data suggest this functional form. A random effect of individual fish was added to the model to account for the repeated measures on the same individuals. All models were estimated with the gam function from the mgcv package of the R language (Wood 2017). From the estimated models, the marginal means of the cumulative capsule intake were computed for each hour and peptide dose. For these marginal means, the previous injection was set to the saline to adjust for the effect of the previous dose and to compare the doses at a similar injection history. In addition, contrasts between the peptide dose and the saline injection were computed for each hour and dose. These contrasts were expressed as ratios against the capsule intake on the saline and they directly estimate the proportional effect of the peptide injection dose on the cumulative capsule intake in relation to the intake on the saline treatment. The marginal means and contrasts were estimated with the emmeans package of the R language (Lenth 2024). Finally, the marginal means and contrasts were presented graphically by plotting the median estimate and its 95 % confidence intervals by hour and dose.

## 3. Results

### 3.1. The effects of Cck on the voluntary feed intake of rainbow trout

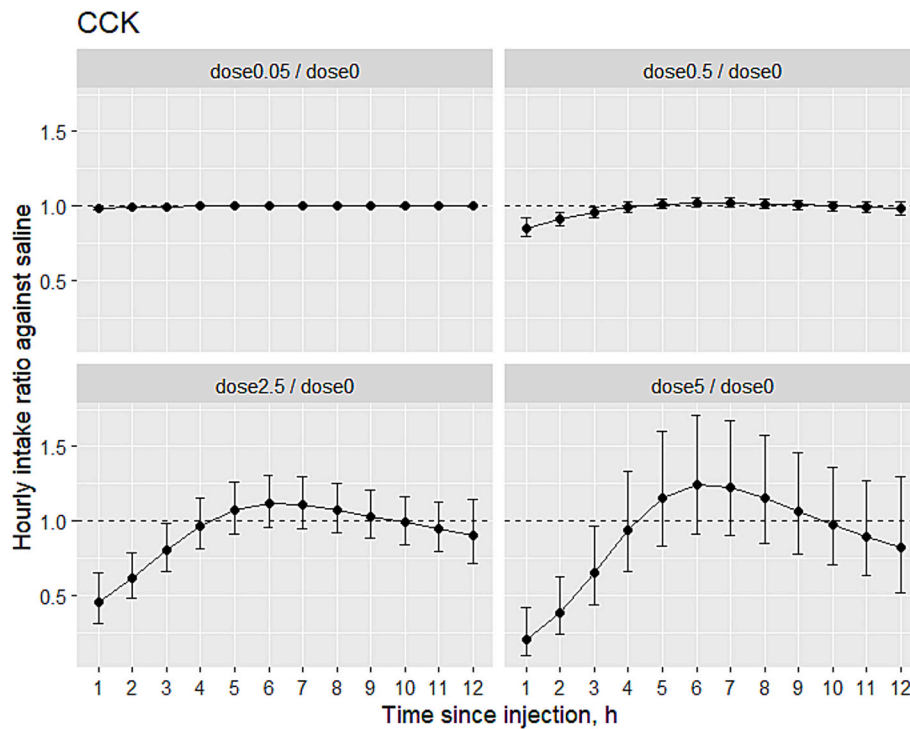
Cck treatment caused a rapid and significant inhibition of feed intake, from the D0.5 to the D5 dose (Fig. 2) at one hour post injection. The inhibition was dose-dependent between doses D0.5 and D2.5, with significant differences between D0.05 and D0.5, as well as between D0.5 and D2.5. The effect did not differ between D2.5 and D5. At all effective doses, the inhibitory effect of Cck diminished rapidly with time, lasting two to three hours. Over the 12-hour observation period, the inhibitory effect of Cck on the over-all feed intake was negligible (Fig. 3), but it is notable that the variation in hourly feed intake increased with increasing Cck dose (Fig. 2). See Table 3 for the statistical modeling applied to the data.

### 3.2. The effect of Grp on the voluntary feed intake of rainbow trout

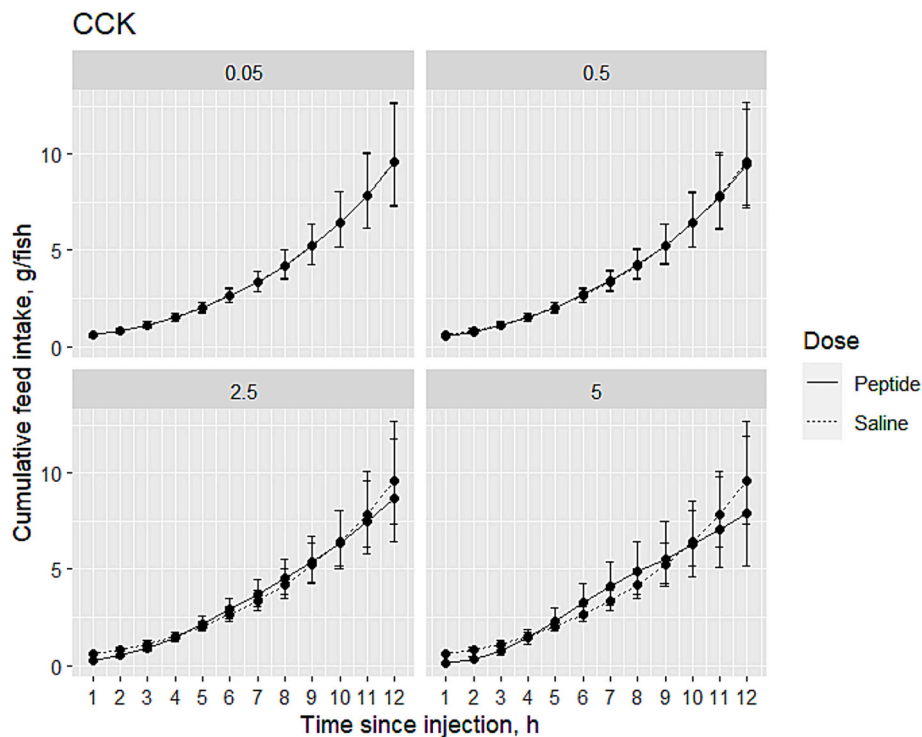
Grp treatment caused a rapid and significant inhibition of feed intake, from the D0.05 to the D5 dose (Fig. 4) at one hour post injection. The inhibition was dose-dependent, with significant differences between D0.05 and D0.5, as well as between D0.5 and D2.5 but not between D2.5 and D5. At all doses, the inhibitory effect of Grp decreased gradually four hours after injection, but the feed intake remained at a lower level as compared to saline throughout the 12-hour observation period. The same prolonged effect was seen for all doses although at the lowest Grp dose, the inhibitory effect was marginal after four hours. Over the 12-hour observation period, the inhibitory effect of Grp was detectable at the D2.5 and D5 doses (Fig. 5). Although total feed intake appeared to decrease also at D0.05 and D0.5 (Fig. 5), this inhibitory effect could not be confirmed statistically. See Table 3 for the statistical modeling applied to the data.

## 4. Discussion

Studies on feed intake and/or appetite in salmonid fish are fraught with difficulties which may affect interpretations of the obtained data in various ways (Best et al. 2023; Conde-Sieira et al. 2018). Although rainbow trout is domesticated to a degree, handling causes stress reactions that are manifested by an inhibition of feeding (Pankhurst et al. 2008). However, a direct approach to examine the role of a hormone in the regulation of feed intake in fish is to submit fish to hormone treatments *in vivo* and to assess feed intake as Hoskins and Volkoff (2011) have noted. This study was performed using a direct method minimizing



**Fig. 2.** The effect of Cck doses (nmol/kg fish) on feed intake in relation to time and saline with a 95% confidence interval. The difference between Cck doses and saline is estimated for each hour and shown as a relative value, where a ratio value of one means there is no difference. Saline is denoted by a dashed horizontal line, and the relative peptide effect as solid line with error bars. Ratio values over one indicates the effect of increasing Cck dose increased feed intake and values below one indicates the effect of increasing dose reduced feed intake relative to saline. Cck effect can be considered different from the saline when the 95% confidence interval is not overlapping the dashed line of saline.



**Fig. 3.** The effect of Cck doses on cumulative (g/fish kg) feed intake. The feed intake is presented in grams per fish by multiplying the eaten capsule amounts by the weight of the capsule (435 mg). The effect of different Cck doses (nmol/kg fish) on feed intake is compared to saline with a 95% confidence interval. The cumulative intake for Cck can be considered different from that of the saline when the confidence interval of Cck does not overlap with the mean of the saline intake.

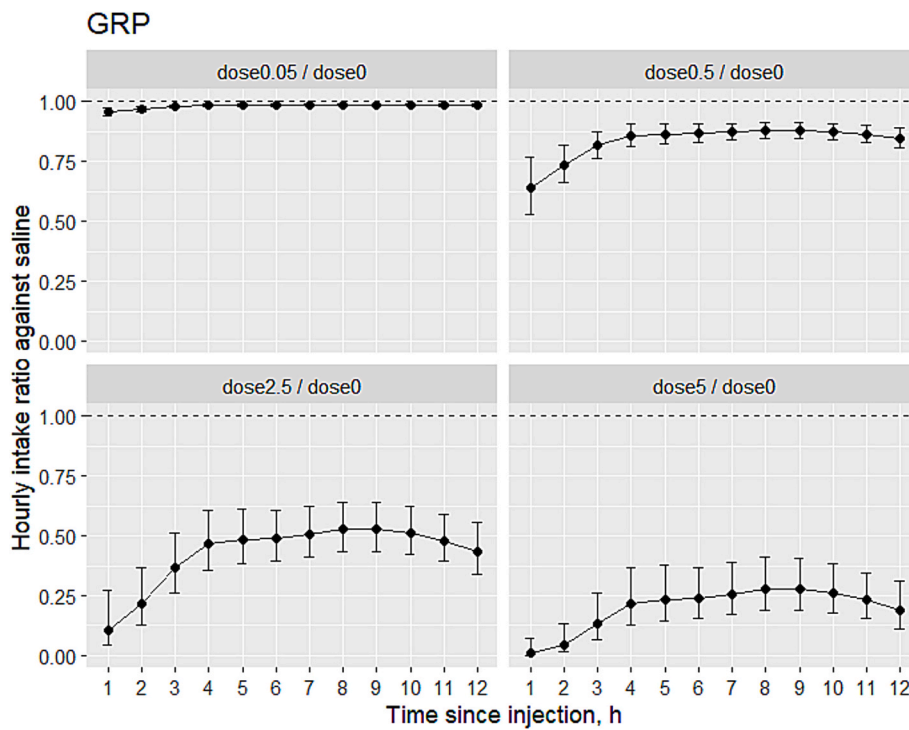
**Table 3**

The statistics of models of the feed intake with CCK and GRP injections. Feed intake is modeled as the number of capsules eaten in each hour. The estimated dispersion parameter of the negative binomial distribution was 1.405 with CCK and 1.33 with GRP.

Component	Term	CCK				GRP			
		Estimate	Std Error	t-value	p-value	Estimate	Std Error	t-value	p-value
A	intercept	0.345	0.092	3.747	0.0002	-0.196	0.096	-2.048	0.0405
	time	0.229	0.016	14.634	0.000	0.293	0.015	19.610	0.000
Component	Term	edf	Ref. df	F-value	p-value	edf	Ref.df	F-value	p-value
	B	time:dose	4.785	5.691	37.658	0.000	5.879	6.958	295.365
	time:prev:	4.420	5.251	47.243	0.000	5.346	6.358	73.020	0.000
	dose								
	time, fish	15.173	17.000	146.145	0.000	15.531	18.000	117.138	0.000

Adjusted R-squared: 0.324, Deviance explained 0.444  
-REML: 3554.660, Scale est: 1.000, N: 1224

Adjusted R-squared: 0.341, Deviance explained 0.542  
-REML: 3390.838, Scale est: 1.000, N: 1332



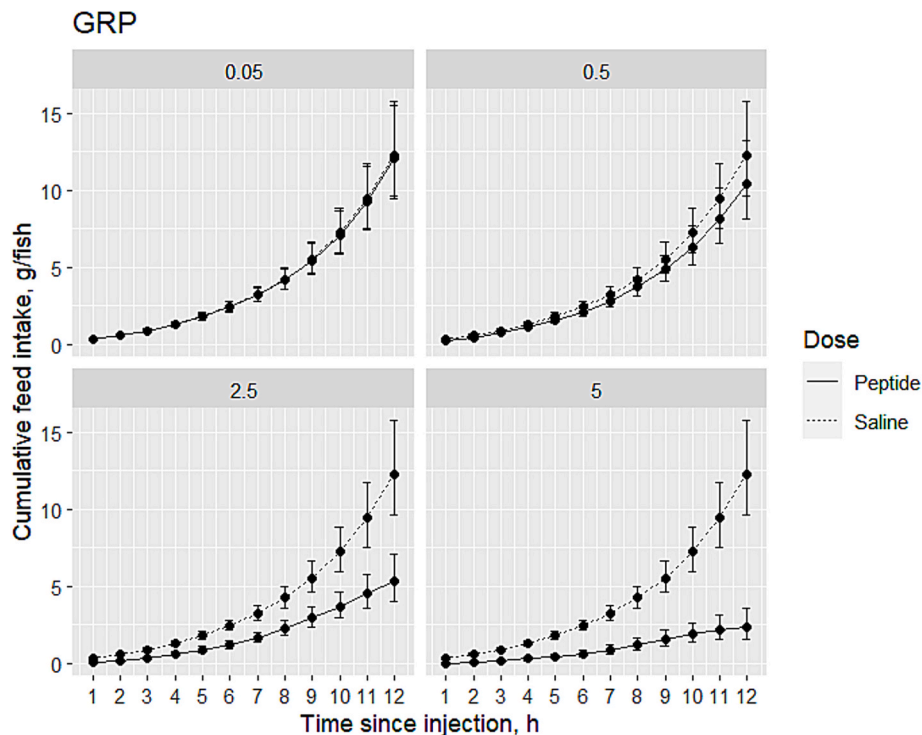
**Fig. 4.** The effect of Grp doses (nmol/kg fish) on feed intake in relation to time and saline with a 95% confidence interval. The difference between Grp doses and saline is estimated for each hour and shown as a relative value, where a ratio value of one means there is no difference. Saline is denoted by a dashed horizontal line, and the relative peptide effect as solid line with error bars. Ratio values over one indicates the effect of increasing Grp dose increased feed intake and values below one indicates the effect of increasing dose reduced feed intake relative to saline. Grp effect can be considered different from the saline when the 95% confidence interval is not overlapping the dashed line of saline.

the stress-induced changes by acclimating the fish to the steady and separate experimental conditions with regular light rhythm and administering the hormone via intraperitoneal cannula, without the need of handling the fish. Monitoring of feed intake was carried out accurately and without disturbing the fish by using a fully automated self-feeding system and floating feed. As high-lipid diets lead to elevated postprandial Cck secretion in rainbow trout (Jönsson et al. 2006), this was taken into account in the experimental design, both in terms of the length of the postprandial period studied and Cck doses used.

The current study demonstrates that Cck has an appetite-inhibiting effect in rainbow trout. This is in line with several studies on fish species (Himick and Peter, 1994a; Rubio et al. 2008; Zhang et al. 2017), as well as in other vertebrate species (Cawthon and de la Serre, 2021), indicating a well conserved function for Cck through vertebrate evolution. The current data show that the Cck-induced appetite suppression lasts for two to three hours. This, together with our earlier data, showing

postprandial elevation of plasma Cck levels in rainbow trout (Jönsson et al. 2006), strongly indicates that Cck has an important function as a postprandial satiety signal in rainbow trout. While the current data show the Cck effect to be dose-dependent, with stronger inhibition of appetite at higher doses, the Cck injections did not significantly affect the 12-hour cumulative feed intake of the rainbow trout. This agrees with the hypothesis by Raybould (2007), based on mammalian studies, that the appetite-suppressing effect of Cck is related to meal termination, but not affecting total daily feed intake. Thus, Cck appears to act as meal terminating factor in rainbow trout and that the signal strength (postprandial plasma Cck levels) could define the length of the inter-meal period.

The current study demonstrates that Grp has a strong, dose-dependent inhibitory effect on appetite in rainbow trout. This is in line with studies on some other fish species (Himick and Peter 1994b, Schroeter et al 2015). The current study shows that even the lowest dose



**Fig. 5.** The effect of Grp doses on cumulative (g/fish kg) feed intake. The feed intake is presented in grams per fish by multiplying the eaten capsule amounts by the weight of the capsule (435 mg). The effect of different Grp doses (nmol/kg fish) on feed intake is compared to saline with a 95 % confidence interval. The cumulative intake for Grp can be considered different from that of the saline when the confidence interval of Grp does not overlap with the mean of the saline intake.

(D0.05) causes a decrease in appetite but does not significantly affect the daily intake of feed. At higher Grp doses, however, the inhibition of voluntary feed intake lasted throughout the 12-hour observation period and thus decreased the daily feed intake. Grp shares close structural homology with bombesin which has similar physiological effects as Grp in a range of species (Merali et al. 1999) including fishes (Volkoff et al. 2000). It has been speculated that the inhibitory effect of bombesin (and Cck8) on feed intake in chicken is caused by activation of gastrointestinal motility, leading to abdominal discomfort (Savory 1987). High doses of bombesin decrease feed intake in rats as they develop aversion to feed associated to the treatment (Deutsch and Parsons 1981; Vanderweele et al. 1985), while no such reaction was noted at lower bombesin doses (Vanderweele et al. 1985). Based on these and other data, Lee et al. (1994) hypothesized that low bombesin levels inhibit appetite by signaling satiety, but at higher levels, it suppresses feeding through the development of feed aversion. Our results fit this hypothesis, as low Grp doses had an appetite-inhibiting effect, while the effect of high Grp doses was strong and long-lasting, which could indicate a non-physiological reaction. Although the results show that Grp affects feed intake in rainbow trout, previous study that found that plasma Grp levels were unaffected by feed intake (Jönsson et al. 2006) must be considered. Thus, while Grp appears to have a potent appetite-inhibiting effect in rainbow trout, it appears not to function as postprandial, short-term satiety signal, as can be concluded for Cck.

The control of feeding behavior is a remarkable multifaceted phenomenon, and although general patterns of fish appetite regulation are known, the information on fish appetite regulation is still limited (Assan et al. 2021). The results of this study, together with previous results (Jönsson et al. 2006), demonstrate a model for how Cck acts as a postprandial satiety signal in rainbow trout. Grp inhibitory effects are also evident in rainbow trout, but the significance as a post-meal satiety signal in rainbow trout remains open and requires further research.

#### CRediT authorship contribution statement

**Antti Forsman:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation. **Elisabeth Jönsson:** Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Björn Thrandur Björnsson:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Katja Anttila:** Writing – review & editing. **Kari Ruuhonen:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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