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RESEARCH ARTICLE

Effects of feeding on *in vivo* motility patterns in the proximal intestine of shorthorn sculpin (*Myoxocephalus scorpius*)

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ABSTRACT

This is the first study to catalogue the diverse array of *in vivo* motility patterns in a teleost fish and how they are affected by feeding. Video recordings of exteriorised proximal intestine from fasted and fed shorthorn sculpin (*Myoxocephalus scorpius*) were used to generate spatio-temporal maps to portray and quantify motility patterns. Propagating and non-propagating contractions were observed to occur at different frequencies and durations. The most apparent difference between the feeding states was that bands of relatively high amplitude contractions propagating slowly in the anal direction were observed in all fasted fish (*N*=10) but in only 35% of fed fish (*N*=11). Additionally, fed fish displayed a reduced frequency (0.21±0.03 versus 0.32±0.06 contractions min^{-1}) and rhythmicity of these contractions compared with fasted fish. Although the underlying mechanisms of these slow anally propagating contractions differ from those of mammalian migrating motor complexes, we believe that they may play a similar role in shorthorn sculpin during the interdigestive period, to potentially remove food remnants and prevent the establishment of pathogens. 'Ripples' were the most prevalent contraction type in shorthorn sculpin and may be important during mixing and absorption. The persistence of shallow ripples and pendular movements of longitudinal muscle after tetrodotoxin (1 μmol l^{-1}) treatment suggests these contractions were myogenic in origin. The present study highlights both similarities and differences in motility patterns between shorthorn sculpin and other vertebrates, as well as providing a platform to examine other aspects of gastrointestinal functions in fish, including the impact of environmental changes.

KEY WORDS: Gastrointestinal, Migrating motor complexes, Ripples, Peristalsis, Propagating, Standing contractions, Fasting, Fed, Spatio-temporal maps, TTX, Fish, Teleost

INTRODUCTION

Maintenance and operation of the gastrointestinal tract are essential for all animals to store, process and assimilate food, as well as providing a barrier against pathogens (Cain and Swan, 2011). External environmental factors are likely to affect gastrointestinal functions, especially in fish, where the gastrointestinal tract is directly exposed to the surrounding water during drinking or eating. To be able to evaluate the impact of environmental factors (e.g. climate change) on fish, it is important to understand how the gastrointestinal tract operates under normal conditions.

Gastrointestinal motility aids in the breakdown of food particles by mixing them with digestive enzymes and then moving the contents at an appropriate rate along the gut to allow absorption of nutrients and expulsion of waste. Motility is achieved through contractions and relaxations of smooth muscle in the gut wall (Kunze and Furness, 1999) and is controlled through coordinated interactions between smooth muscle, interstitial cells of Cajal (ICC), hormones and the autonomic nervous system (Olsson and Holmgren, 2001). Contractions can be either non-propagating (standing) or propagating, and depending on the frequency, duration, velocity, direction and amplitude of the contraction types, different patterns can be distinguished. Mammalian motility patterns are well known and have been studied for over 100 years (Bayliss and Starling, 1899; Boldyreff, 1902; Cannon, 1902); however, significantly less is known about motility patterns in fish.

In mammals, motility patterns can be confined to a particular region or encompass large sections of the gut (Szurszewski, 1969). Often, different patterns dominate in different regions of the gut, but this may also depend on the fed state of the animal (Ailiani et al., 2009; Berthoud et al., 2002; Bogeski et al., 2005; Chen et al., 2013; D'Antona et al., 2001; Ferens et al., 2005; Hennig et al., 1997; Hennig et al., 1999; Hennig et al., 2010). Mixing and propulsion can be achieved by either non-propagating (e.g. alternating standing contractions during segmentation) or propagating contractions (e.g. peristalsis and ripples) (Chen et al., 2013; D'Antona et al., 2001; Hennig et al., 2010; Huizinga et al., 2011). Interdigestive motility patterns are generally characterised by migrating motor complexes (MMCs), which slowly propagate uninterrupted along the intestine for relatively long distances (Deloose et al., 2012; Husebye, 1999; Szurszewski, 1969). In many mammals, MMCs are disrupted after food intake, with motility patterns reverting to non-propagating segmentation and propagating peristaltic contractions to mix and propel food contents along the tract (Husebye, 1999). However, especially in continuous feeders, MMCs often persist after food intake (Buéno and Ruckebusch, 1976; Ruckenbusch and Buéno, 1976).

Data on gut motility in fish are limited and stem almost exclusively from *in vitro* studies on isolated gut sections or muscle strip preparations (Gräns and Olsson, 2011; Olsson, 2011a). While propagating and standing contractions have been identified *in vitro* in, for example, brown trout (*Salmo trutta*), lesser-spotted dogfish (*Scyliorhinus canicula*) and Atlantic cod (*Gadus morhua*) (Andrews and Young, 1993; Burnstock, 1958a; Burnstock, 1958b; Karila and Holmgren, 1995), they may not necessarily reflect *in vivo* patterns (Bush et al., 2000; Fox et al., 1983; Fujimiya and Inui, 2000; Sarna, 1991). So far, the only *in vivo* studies involve larval zebrafish (*Danio rerio*) and halibut (*Hippoglossus hippoglossus*), identifying anterograde and retrograde propagating contractions (Holmberg et al., 2007; Holmberg et al., 2006; Holmberg et al., 2003; Holmberg et al., 2004; Ronnestad et al., 2000). However, as few *in vivo* motility studies are performed on conscious animals, it should be kept in mind that some anaesthetics may suppress or modify normal motility patterns (Buéno et al., 1978; Fujimiya and Inui, 2000).

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List of abbreviations

cpm	contractions per minute
EEA	enteric electrical activity
ICC	interstitial cells of Cajal
MMC	migrating motor complex
ST	spatio-temporal
TTX	tetrodotoxin

Nonetheless, motility patterns (e.g. MMCs and peristalsis) observed in conscious animals (Hellström and Ljung, 1996; Ruckebusch and Buéno, 1976; Szurszewski, 1969), have also been documented in several anaesthetised animals (Healy et al., 1981; Krantis et al., 1998; Lammers et al., 2005; Wright et al., 1982).

In the present study, shorthorn sculpin, *Myoxocephalus scorpius* (Linnaeus 1758), were used to study gastrointestinal motility patterns in adult fish. Shorthorn sculpin are widespread in the northern hemisphere (Scott and Scott, 1988), but individuals are believed to be quite stationary, making them good models to evaluate the effects of environmental changes (Dick et al., 2009). They are a benthic marine species described as opportunistic ambush predators (Dick et al., 2009; Moore and Moore, 1974; Scott and Scott, 1988; Seth and Axelsson, 2009) and have a gastrointestinal system consisting of a U-shaped sac-like stomach, pyloric caeca and a relatively short intestine (Olsson, 2011b). Because of their feeding strategy (i.e. intermittent feeding and carnivorous diet), ease of capture and survivability in captivity, shorthorn sculpin were selected as a model animal for this study.

Spatio-temporal (ST) maps generated from video recordings of the gastrointestinal tract have been used in numerous mammalian studies to examine gastrointestinal movements (Hennig et al., 1997). The ST maps can be used to visualise and quantitatively describe complex motility patterns, as they allow identification of a range of different contraction types as well as their respective spatial (regional distribution) and temporal (frequency, duration, speed, etc.) characteristics. Hence, the aim of the present study was to use video recordings and ST maps to describe motility patterns in the proximal intestine of anaesthetised adult fish. Additionally, we investigated whether differences occur between interdigestive (fasted) and postprandial (fed) motility patterns in shorthorn sculpin.

RESULTS

Video recordings and ST maps from the proximal intestine of both fasted and fed adult shorthorn sculpin (*M. scorpius*) showed a wide range of changes in intestinal diameter, reflecting contractions and relaxations (see Materials and methods for a detailed description). The contractile activity could be classified as either propagating or non-propagating. Depending on the velocity, frequency, duration and amplitude of the contractions, different types of contractions were discerned: ripples, slow anally propagating contractions, long-duration orally propagating contractions, standing contractions and longitudinal contractions (Fig. 1).

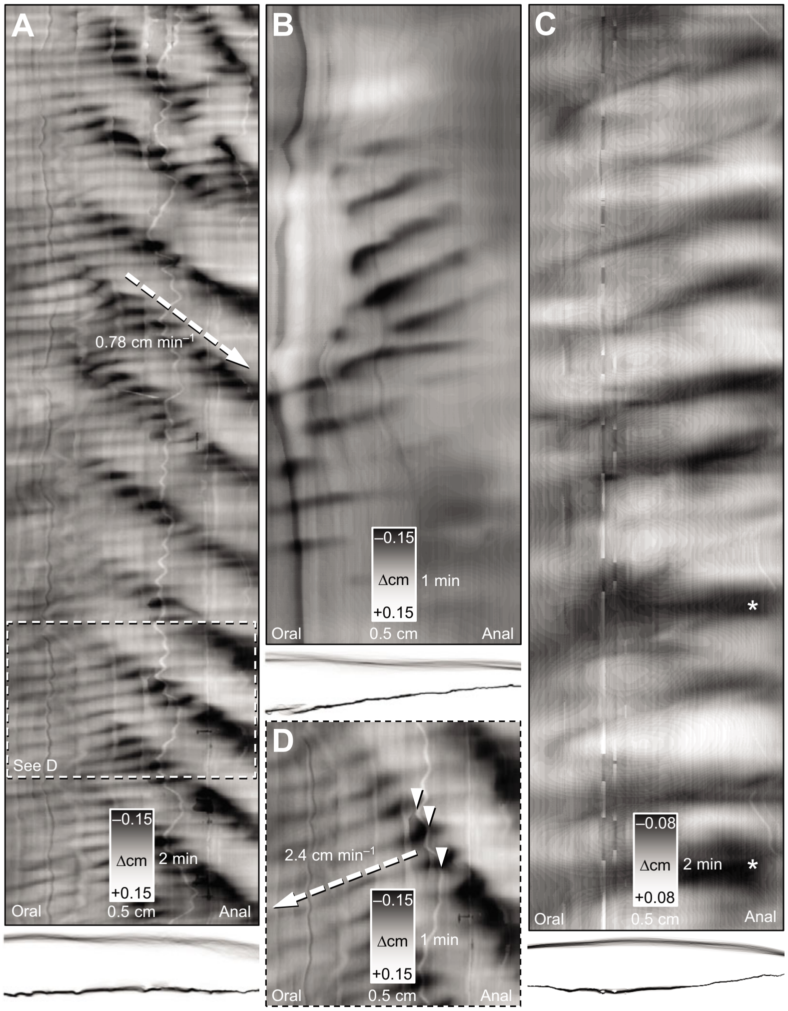

Fig. 1. Spatio-temporal maps (ST maps) displaying examples of the different types of contractions observed in the proximal intestine of shorthorn sculpin. The ST map displays the diameter (portrayed as a greyscale value with black pixels representing contractions and white pixels representing dilations) of the proximal intestine along the exteriorised section (distance: horizontal axis) and during the recording period (time: vertical axis). The image at the bottom of each ST map shows the overall outline during the recording period (see Materials and methods for description). (A) Ongoing orally propagating contractions, which are characterised as ripples (visualised as narrow, black lines on the ST map), were common in both fasted and fed fish; however, slow anally propagating contractions (visualised as broader black bands on the ST map; see dashed arrow) were more prevalent in fasted fish. (B) Ripples were also observed to occur in brief trains followed by a period of quiescence. (C) Standing or fast-propagating contractions (horizontal black bands denoted by asterisks) and the associated dilations either side of the contraction along the exteriorised segment (white areas) were more prevalent after feeding. (D) Magnified view showing longitudinal movements (arrowheads) associated with ripples (dashed arrow) and slow anally propagating contractions.

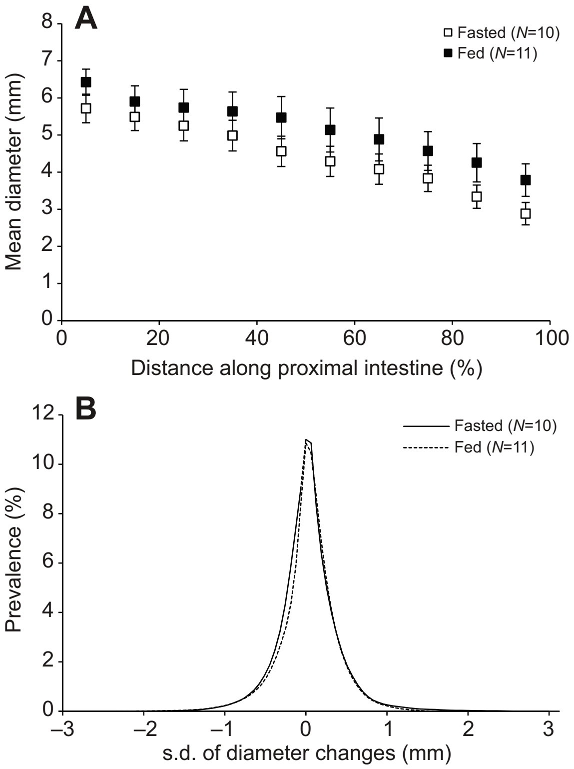

Fig. 2. Mean diameter and contractility of the proximal intestine in fasted (*N*=10) and fed (*N*=11) shorthorn sculpin. (A) Mean (±s.e.m.) diameter of the proximal intestine at different positions along the section. (B) The prevalence of different strength contractions throughout the 90 min experimental period in both fasted and fed shorthorn sculpin. Differences in mean diameter and prevalence between fasted and fed fish were not significant.

Only fish with stable heart rate and blood flow values throughout the entire experimental period were included in the analysis (fasted: 10/12; fed: 11/13). The mean heart rate (31.5±2.5 beats min^{-1}) during the experimental period did not significantly differ (*P*=0.108, *t*=1.683, d.f.=20) from values (32.4±2.2 beats min^{-1}) measured prior to accessing the proximal intestine.

The slightly larger mean diameter of the proximal intestine seen in fed (5.2±0.3 mm) compared with fasted fish (4.4±0.3 mm; Fig. 2A) is probably due to the presence of food in the former (visualised as dark matter in contrast to the lighter coloured intestine). In both groups, mean diameter decreased from the oral to the anal end of the proximal intestine (fasted: 5.7 to 2.9 mm, fed: 6.4 to 3.8 mm). Fig. 2B shows the proportion of time (prevalence) any region of the proximal intestine was at a particular state of contraction. A best-fit Gaussian curve was compared with the data to estimate the s.d. of the diameter changes. There was no significant difference (*P*=0.628, *t*=0.492, d.f.=19) in the s.d. of diameter changes between fasted (0.28±0.04 mm) and fed fish (0.26±0.04 mm), meaning the two groups displayed similar proportions of large and small contractions. However, clear differences in the overall motility patterns were observed between fasted and fed shorthorn sculpin.

Ripples

The most ubiquitous propagating contractions in both fasted and fed shorthorn sculpin were rhythmic, shallow circular muscle contractions (Fig. 1A), which based on their appearance will subsequently be referred to as 'ripples' (D'Antona et al., 2001). Ripples were observed, albeit with some variability of prevalence, in all of the fish studied. The contractions were highly rhythmic in most individuals, although periods with distinct contractions were often interspersed with periods of weaker activity, in which the frequency and amplitude of contractions were substantially lower (Fig. 1A). This waxing and waning varied substantially between individuals.

Figs 3 and 4 show a range of motility parameters for ripples. The ripples were shallow, with the majority (>75%) having an amplitude equal to or less than a 15% decrease in the mean diameter at the point where the contraction occurred (Fig. 3A). During periods of distinct and rhythmic contractions, the time intervals between successive ripples were fairly constant, ranging from 20 to 30 s (Fig. 3B). Initiation of ripples was not restricted to a specific region of the gut, but could occur throughout the proximal intestine (Fig. 4A). However, a significantly (*P*=0.002, *t*=3.552, d.f.=19) higher proportion of ripples were initiated in the first half of the proximal intestine in fasted (mean: 75±6%) compared with fed fish (mean: 42±7%). Approximately 95% of ripples propagated in a retrograde direction (Fig. 4B), which, combined with the location of initiation sites, resulted in a bias for the prevalence of ripples to be greater in the oral half of the proximal intestine in both treatments

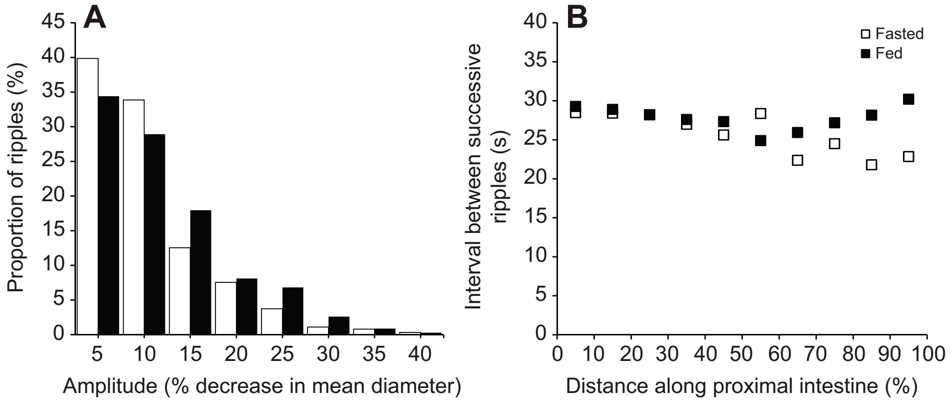

Fig. 3. Amplitude of ripples and time interval between successive ripples in fasted (*N*=10) and fed (*N*=11) shorthorn sculpin. Contraction amplitude (A) was converted from mm to a relative measure in relation to the mean diameter of the specific section of proximal intestine where the ripple occurred. This was done to account for the differences in average diameter between individual fish as well as between different sections of the proximal intestine. All measures are mean values calculated from the total 90 min experimental period for fasted and fed fish. For calculation of the interval between ripples (B), the position along the proximal intestine was normalised for each fish. No significant differences were observed between the two treatments.

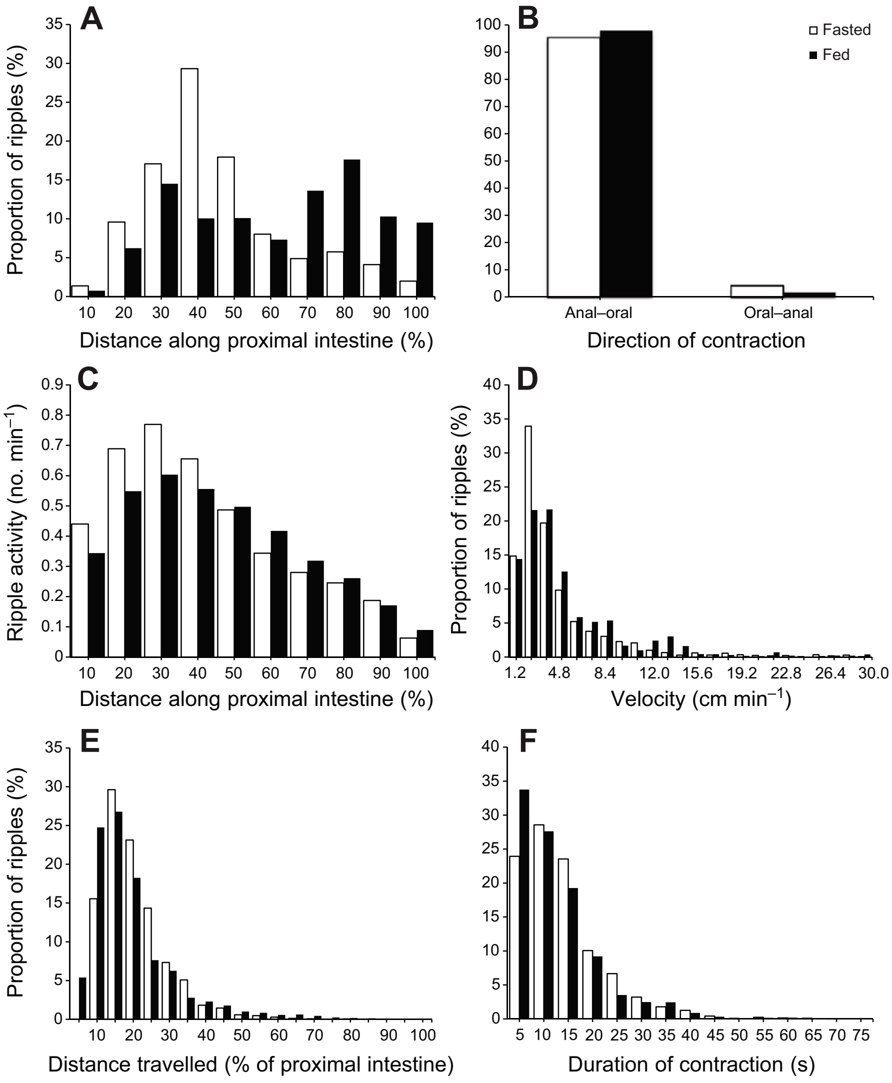

Fig. 4. Motility parameters for ripples in fasted (*N*=10) and fed (*N*=11) shorthorn sculpin. (A) Initiation sites of ripples along the proximal intestine (a significantly higher proportion of ripples was initiated in the first half of the intestine in fasted fish, *P*=0.002). (B) Direction of propagation. (C) Distribution of ripple activity along the proximal intestine. (D) Velocity of ripples. (E) Distance travelled by ripples. (F) Duration of contractions. Data are presented as mean values calculated from the total 90 min experimental period. The position along the proximal intestine was normalised for each fish. With the exception of differences in the distribution of initiation sites between fasted and fed fish, there were no significant differences in the motility parameters of ripples between the two treatments.

(Fig. 4C). The median velocity of ripple propagation was similar in fasted (3.3 cm min^{-1}) and fed (3.9 cm min^{-1}) fish (Fig. 4D). The majority of the ripples (~90%) propagated a distance of less than 30% of the length of the proximal intestine (median distance: 18% and 17% in fasted and fed fish, respectively, Fig. 4E) and with an average duration of less than 25 s (median: 12 and 10 s, respectively, Fig. 4F).

Slow anally propagating contractions

A second type of rhythmic propagating contractions observed in shorthorn sculpin were more prolonged, circular muscle contractions, which slowly propagated in an anterograde direction over a large proportion of the proximal intestine (Fig. 1A,D).

Compared with ripples, these contractions were on average 3–4 times slower in both fasted (median velocity: 1.0 cm min^{-1}) and fed (median velocity: 0.9 cm min^{-1}) fish (Fig. 5A). Furthermore, they propagated on average 2–3 times further (mean: 56±3% and 46±4% of the section in fasted and fed fish, respectively, Fig. 5B) and had contraction durations ~10 times longer than ripples (mean: 129±19 and 115±14 s in fasted and fed fish, respectively, Fig. 5C). As in ripples, there were multiple initiation sites for slow anally propagating contractions and a significantly (*P*=0.032, *t*=2.427, d.f.=12) higher proportion were initiated in the first half of the proximal intestine in fasted (mean: 82±8% of contractions) compared with fed fish (mean: 47±12% of contractions) (Fig. 5D).

Similar to ripples, slowly propagating contractions were relatively rhythmic but periods of interruptions were not uncommon. For the majority of contractions, the time between successive contractions ranged between 80 and 160 s (Fig. 5E). The mean amplitude of the contractions was on average three times greater than that of ripples, resulting in a mean decrease in the diameter of the proximal intestine of 31±1% and 33±1% for fasted and fed fish, respectively (Fig. 5F). However, there was some variation between and within individual fish. Almost two-thirds of these propagating contractions were of medium amplitude (between 20% and 40% decrease in resting diameter), while low amplitude (<20% decrease in resting diameter) and high amplitude contractions (>40% decrease in resting diameter) each accounted for ~20% of these contractions. As these contractions propagated anally, they were observed to enhance the amplitude of ripples. This effect varied depending on the amplitude of slow anally propagating contractions: low amplitude contractions barely modulated the amplitude of underlying ripples, medium amplitude contractions had a mixture of sustained or nearly sustained contraction at the leading edge and ripples appeared more intensely, and high amplitude contractions had a sustained contraction and underlying ripples could no longer be observed (Fig. 6).

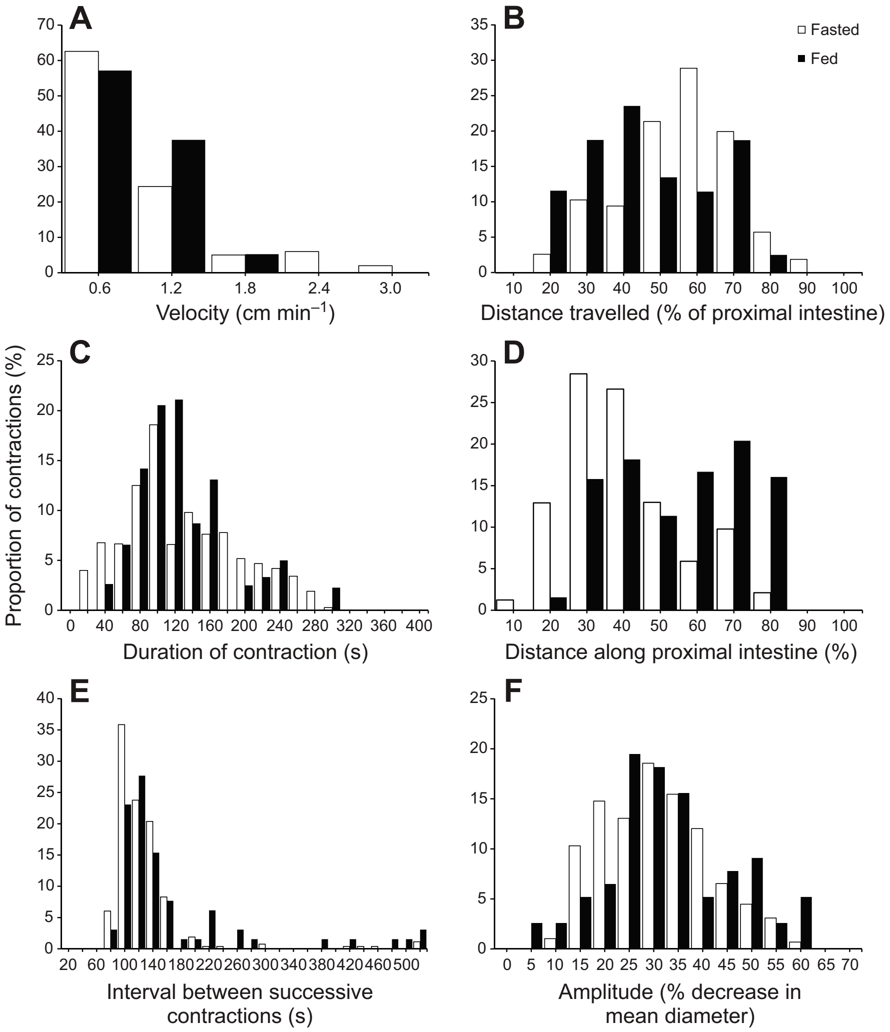

Fig. 5. Motility parameters for slow anally propagating contractions in fasted (*N*=10) and fed (*N*=4) shorthorn sculpin. (A) Velocity. (B) Distance travelled along the proximal intestine. (C) Duration of contractions. (D) Distribution of initiation sites (a significantly higher proportion of contractions was initiated in the first half of the intestine in fasted fish, *P*=0.032). (E) Time interval between successive contractions. (F) Amplitude of contractions. Data are presented as mean values calculated from the total 90 min experimental period. Sample size for the fed treatment was lower as fish that did not display these contractions were excluded. The position along the proximal intestine was normalised for each fish. With the exception of differences in the distribution of initiation sites between fasted and fed fish, there were no significant differences in the motility parameters of these contractions between the two treatments.

Long duration, orally propagating and standing contractions

A third type of propagating contractions was observed in six out of 21 fish (two fasted, four fed). In contrast to ripples and slow anally propagating contractions, they were relatively rare and without any apparent rhythmicity when present. They propagated orally over a large proportion (median: 60% of the section) of the proximal intestine (Fig. 1C). The mean amplitude of these contractions was relatively similar to that of slow anally propagating contractions with a mean decrease in the diameter of the proximal intestine of 26±1%. The contractions propagated at an intermediate velocity (median: ~1.8 cm min^{-1}) compared with ripples and slow anally propagating contractions.

Standing contractions were generally large amplitude (>50% decrease in the mean diameter of the proximal intestine), and occurred almost simultaneously so that calculation of their velocity was difficult. These large amplitude contractions were often accompanied by dilations on either side of the contraction (Fig. 1C). These contractions were observed in most shorthorn sculpin (17 out of 21 fish); however, their appearance was without any apparent rhythmicity. The contractions also varied substantially within an individual as well as between individuals in parameters such as duration of contraction (ranging between 5 and 100 s) and the proportion of proximal intestine that simultaneously contracted (ranging between 5% and 50% of the length of the proximal intestine).

Longitudinal contractions

In addition to the circular muscle contractions, rhythmic pendular contractions of the longitudinal muscle were observed in all of the preparations and were visible as oscillating lines in ST maps as a result of a side-to-side movement of the proximal intestine (Fig. 1D). As observed from the ST maps, larger longitudinal displacements tended to occur at the same time as slow anally propagating contractions, whereas in the absence of such contractions the longitudinal contractions were much smaller. As we did not specifically mark points along the intestine, quantifying longitudinal movements was unreliable.

Effects of feeding on motility patterns

Although most of the contraction types could be seen in both fed and fasted fish, the frequencies differed, resulting in clearly different overall motility patterns between the groups (Fig. 6). Generally, the overall motility pattern of fasted fish can be characterised by rhythmically occurring, slow anally propagating contractions and retrograde ripples (Fig. 6A–D), whereas, less rhythmicity in the motility patterns of fed fish was observed, with a higher degree of standing contractions in the absence or presence of ripples (Fig. 6E–H).

Feeding seemed to reduce the prevalence of rhythmic slow anally propagating contractions. While all fasted fish (*N*=10) displayed this type of contraction, only four fed fish (*N*=11) did. A Chi-square test demonstrated a significant and moderately strong association between

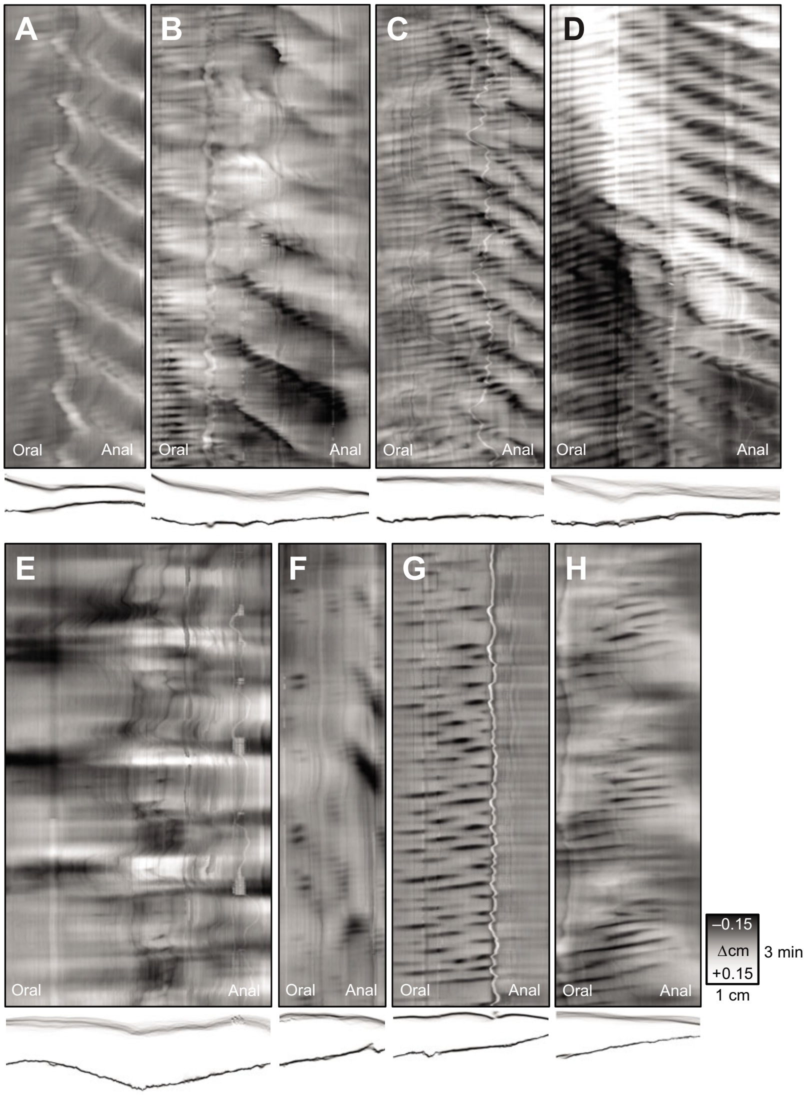

Fig. 6. Examples of ST maps from individual fasted and fed fish displaying clear differences in overall motility patterns between the two treatments. Fasted fish (A–D) had motility patterns predominantly consisting of ongoing, slow anally propagating contractions throughout the recording period (visualised, depending on the individual, as 12–16 broad bands on the ST maps) and retrogradely propagating ripples, whereas fed fish (E–H) displayed a significantly reduced frequency and rhythmicity of slow anally propagating contractions and instead displayed more standing contractions with or without ripples throughout the recording period. There was a significant and moderately strong association between treatment type and presence/absence of slow anally propagating contractions (*P*=0.002). Overall outlines of the exteriorised intestines are displayed below each ST map. Each ST map is 20 min long (vertical axis).

treatment type and presence/absence of slow anally propagating contractions (χ^2_1=9.545, φ=0.674, *P*=0.002). In shorthorn sculpin that displayed these contractions, not only did fasted fish display a higher frequency of contractions per minute (0.32±0.06 cpm) than fed fish (0.21±0.03 cpm) but also there were clear differences in the rhythmicity or periodicity of these contractions. The majority (6/10) of the fasted fish displayed these contractions regularly, one contraction every 118±1 s, throughout the entire recording period (90 min). In contrast, only one fed fish displayed slow anally propagating contractions throughout the entire observation period and the interval between successive contractions seemed to vary more than in fasted fish (146±12 s). Feeding also reduced (*P*=0.031, *t*=2.324, d.f.=19) the frequency of rhythmic longitudinal contractions (mean: 0.48±0.03 and 0.39±0.03 cpm in fasted and fed fish, respectively).

In contrast, feeding increased the frequency of standing contractions. These were observed in all fed (11 out of 11) shorthorn sculpin but only in six out of 10 fasted fish. Furthermore, fed fish had a median frequency of standing contractions of 0.10 cpm, which was significantly higher (Mann–Whitney *U*=15.5, *z*=–2.805, *P*=0.005) than that of fasted fish (0.02 cpm).

Ripples occurred at an average frequency of ~1–2 cpm in both fasted and fed fish and no significant difference was observed in either retrograde or anterograde propagating ripples (Mann–Whitney *U*=45.00, *z*=–0.704, *P*=0.481). However, as already noted, feeding resulted in more variation in the distribution of initiation sites. As mentioned above, long duration orally propagating contractions were relatively rare. In fish displaying this contraction type, the frequency was low (0.1 cpm) and did not differ between treatments (*P*=0.825, *t*=0.234, d.f.=5).

Effects of tetrodotoxin on motility patterns

The addition of tetrodotoxin (TTX; 1 μmol l^{-1}) resulted in altered motility patterns in both fasted and fed fish (Fig. 7). This was most apparent in fed fish where the irregular activity that was normally present disappeared.

Overall, it was also apparent that ripples persisted after the addition of TTX and in most cases (six out of eight fish) increased in frequency throughout the proximal intestine as seen by a reduction in the mean interval between successive ripples (Table 1). In contrast, the amplitude of ripples decreased in response to TTX (Table 1). Furthermore, TTX increased the proportion of anterograde (oral–anal) propagating ripples from ~5% to greater than 40%, while propagation distance, duration of contractions, initiation sites and velocity were not significantly altered (Table 1).

Slow anally propagating contractions were present in all fasted (*N*=4) and two fed fish prior to the addition of TTX. In fasted fish,

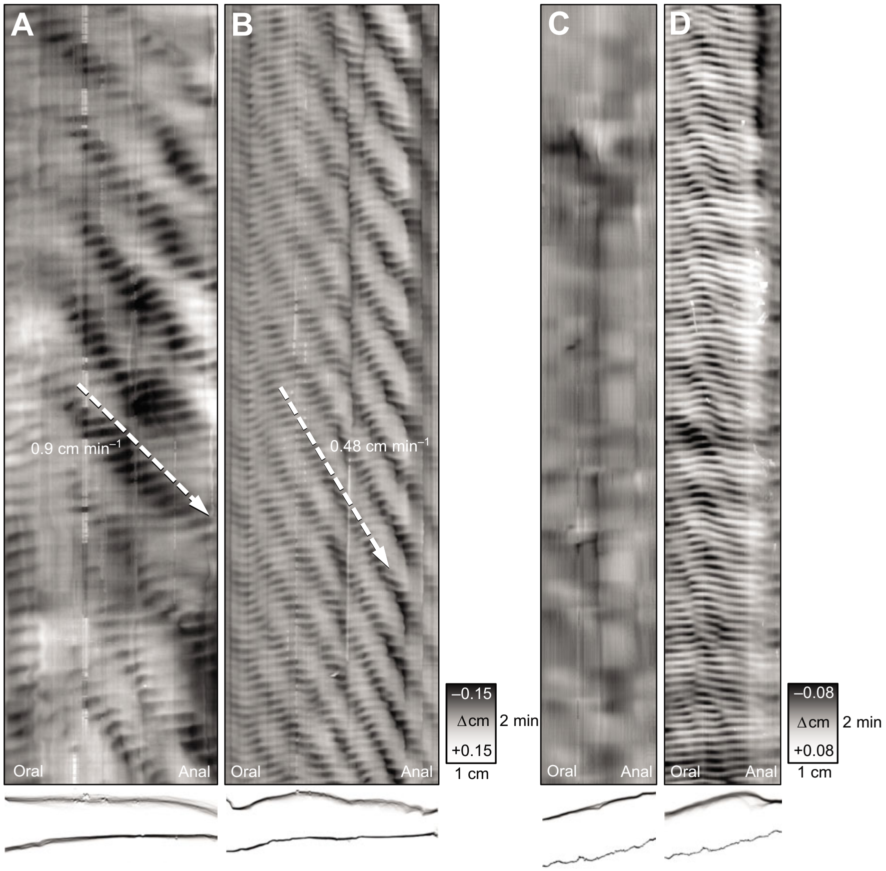

Fig. 7. Examples of ST maps from fasted and fed shorthorn sculpin before and after exposure to tetrodotoxin (TTX, 1 μmol l^{-1}). Exposure to TTX altered motility patterns present in both fasted (A, control; B, TTX) and fed (C, control; D, TTX) shorthorn sculpin. Ripples persisted after addition of TTX in both fed and fasted fish, whereas slow anally propagating contractions (observed predominantly in fasted fish) were reduced in velocity and amplitude (dashed arrows). Standing contractions in fed fish reverted to motor patterns consisting of ripples with slow anally propagating contractions, much like those in fasted fish after TTX treatment. Overall outlines of the exteriorised intestines are displayed below each ST map and each ST map is 20 min long (vertical axis).

these contractions were still visible after TTX addition in three out of four fish, although with markedly reduced velocity and amplitude (Fig. 7A,B). The frequency of the contractions in fasted fish (*N*=3) increased by ~0.2 cpm while velocity decreased to approximately half that of controls. The stability of all contractions was much improved after TTX treatment. TTX treatment in fed fish abolished long duration orally propagating contractions; however, slow anally propagating contractions were still present in two out of four fish with similar characteristics to those observed in fasted fish after TTX (Fig. 7C,D).

DISCUSSION

Using a newly designed experimental arrangement in which we can study the proximal intestine in an anaesthetised fish, we portrayed and quantitatively analysed characteristics of a range of gastrointestinal motility patterns in shorthorn sculpin (*M. scorpius*). This is the first time such patterns have been described in live adult fish. We have also shown that motility patterns depend on the fed state of the fish. Many patterns displayed by sculpin were reminiscent of, or may have similar functions to, mammalian motor patterns, and included both propagating and non-propagating contractions. While we did not specifically quantify flow of intraluminal contents, many contractions were occlusive or near-occlusive (≥50% o.d.) and are likely to cause significant movement of gut contents (Hennig et al., 1997).

Table 1. Motility parameters for ripples in shorthorn sculpin (*N*=8) before (control) and after exposure to tetrodotoxin (TTX, 1 μmol l^{-1})

	Control	TTX	*P*
Frequency (cpm)	2.1±0.6	4.1±1.4	0.215
Initiated in first half of section (%)	54±16	82±7	0.066
Propagation distance (% of section)	19±2	26±8	0.470
Duration of contraction (s)	11±2	11±2	0.877
Velocity (cm min^{-1})	4.1±0.7	5.5±1.1	0.116
Amplitude (% decrease in mean diameter)	13±1	8±1	0.003*
Interval between succesive ripples (s)	26±1	18±1	0.006*
Propagating anally (%)	4±3	42±15	0.036*

Shorthorn sculpin used for TTX analysis consisted of four fasted and four fed fish. Data are presented as mean (±s.e.m.) values calculated from the 30 min experimental period before and after the addition of TTX.

*P<0.05 compared with control.

Although both standing and propagating contractions have been previously identified in different regions of the gut in fish, these studies are almost exclusively based on *in vitro* recordings of muscle contractions (Andrews and Young, 1993; Burnstock, 1958a; Burnstock, 1958b; Karila and Holmgren, 1995). Severing the gut from its blood supply and extrinsic nervous control systems, as well as from other regions of the gut, probably results in contractile activities different to those occurring naturally (Bush et al., 2000; Fox et al., 1983; Fujimiya and Inui, 2000; Sarna, 1991). Using an exteriorised loop of proximal intestine, as in the present study, is likely to better approximate *in vivo* gut motility patterns as both blood supply and extrinsic innervation are maintained. Furthermore, ST maps generated from video recordings of the intestine made it possible to follow the contractile activity along the gut over long periods of time at high spatial and temporal resolution, allowing propagation of contractions to be quantified in detail.

Fish were anaesthetised during the course of the experiment, which may have altered the frequency, amplitude and propagation speed of the contractions (Buéno et al., 1978; Fujimiya and Inui, 2000). For example, *in vivo* gastrointestinal transit in rats was significantly reduced after the administration of anaesthetic (Ailiani et al., 2009; Torjman et al., 2005), whereas, in pigs, the frequency of slow waves varied depending on the type of anaesthetic used (Tachecí et al., 2013). MS222, the general anaesthetic used in this study, has been shown to impair cardiovascular performance in fish (Hill et al., 2002; Houston et al., 1971). However, mean heart rates of anaesthetised sculpin during the experimental period (31.5±2.5 beats min^{-1}) were similar to those found in free-swimming shorthorn sculpin (33.9±1.5 beats min^{-1}) at similar temperatures (Gräns et al., 2013). As well as stable heart rates, cardiac output also remained relatively stable throughout the experiment, suggesting that the dose of MS222 was at a sufficient level to maintain anaesthesia whilst not significantly impacting cardiovascular performance.

Effect of feeding state on overall motility patterns

Previous studies measuring enteric electrical activity (EEA) in smooth muscle of the gastrointestinal tract in a fish species (Gräns et al., 2009) and in a range of mammals (Fioramonti and Buéno, 1984; Rodríguez-Membrilla et al., 1995; Yin et al., 2004) showed differences between feeding states, with an increased EEA occurring postprandially. As electrical measurements are an indirect way to measure muscular contractions, this suggests that contractile activity increases postprandially. In contrast, the analysis of prevalence in this study showed that the two groups displayed similar degrees of contractile activity, but instead there were significant differences in the patterning of motility between fasted and fed fish. Further investigations correlating EEA with different types of contractions would be useful for future telemetric studies intending to use EEA to describe motility patterns in free-swimming fish.

The most apparent effect after feeding was a reduction in the rhythmic pattern composed of slow anally propagating contractions passing along the intestine approximately every 2–3 min. Motor patterns in fed fish were generally more variable and displayed a higher frequency of standing contractions. These standing contractions occur over much larger areas compared with mammalian motor patterns (e.g. segmentation) (Cannon, 1902; Hennig et al., 2010). Given the large amplitude of these contractions, they are likely to cause significant flow of contents out of the contracting segment into adjacent regions (Husebye, 1999; Kunze and Furness, 1999). Somewhat surprisingly, few, if any, fast anterograde contractions, reminiscent of mammalian peristaltic contractions that propel intraluminal contents along the tract (Hennig et al., 1999; Tonini et al., 1996), were seen in fed fish.

Feeding also slightly reduced the frequency of longitudinal contractions in shorthorn sculpins. The frequency (0.17–0.56 cpm) was much lower than the 32–37 cpm recorded in mammals (Cheung and Daniel, 1980; Hennig et al., 1999) but similar to what Burnstock (Burnstock, 1958b) observed *in vitro* in brown trout (0.5 cpm). Similar to other observations (Burnstock, 1958b; Smith and Robertson, 1998; Yokoyama and North, 1983), the longitudinal muscle contracted in synchrony with the circular muscle during a propagating wave of circular muscle contraction. Longitudinal contractions have been suggested to enhance propulsion or mixing of intestinal contents (Melville et al., 1975), although this does not explain the reduced frequency observed in fed fish.

Long duration orally propagating contractions were rare in both fed and fasted fish, suggesting their role may be small during digestion in fish. Retrograde peristaltic contractions have been observed in other fish species and have been suggested to provide a mechanism by which fish can fill the pyloric caeca (Ronnestad et al., 2000) or retain and mix digestive secretions and chyme in the gut for further digestion and absorption (Andrews and Young, 1993).

Do myogenic ripples represent slow wave activity?

The most common contractions in the proximal intestine of shorthorn sculpin were reminiscent of the ‘myogenic ripples’ described by D’Antona and colleagues (D’Antona et al., 2001). Ripples are the most prevalent contraction type in the small and large intestine of mammals such as guinea pigs, rats and rabbits (Benard et al., 1997; D’Antona et al., 2001; Dinning et al., 2012; Hennig et al., 2010; Huizinga et al., 2011), but this is the first time this contraction type has been described in live adult fish. The role of ripples is not clearly understood, but it has been suggested that they could promote or optimise absorption (Chen et al., 2013; Dinning et al., 2012; Hennig et al., 2010) by mixing/circulating the intestinal contents over the mucosal surface of the gastrointestinal tract (Lee, 1983). The lower frequency and velocity of ripples in shorthorn sculpin compared with mammals can probably be accounted for by differences in temperature (sculpin experiments were performed at 10°C compared with 37°C in most mammalian studies) and thermoregulation strategies (ectothermic versus endothermic) of the respective classes.

Ripples persisted after TTX treatment, indicating that they are not dependent on neural activity for their generation or propagation. In mammals, it has been shown that ripples have a similar frequency to slow waves observed in the smooth muscles of the gastrointestinal tract, ranging between 2 and 30 cpm depending on the species and region of the gastrointestinal tract (Berčík et al., 2000; Ferens et al., 2005; Kobayashi et al., 1996; Yoneda et al., 2002). Slow waves are generated by the ICC, which act as pacemakers in the mammalian gut (Huizinga et al., 1995; Sanders, 1996; Sanders et al., 2006; Ward et al., 2004). ICC-like cells were first demonstrated in a fish species by Kirtisinghe (Kirtisinghe, 1940), but it is not until recently that additional studies on ICC distribution in fish have been published (Rich et al., 2007; Uyttebroek et al., 2013). The distribution of ICC in shorthorn sculpin is so far unknown and no measurements of slow wave activity have yet been carried out in any fish species. The similarities in appearance and rhythmicity of ripples in shorthorn sculpin to those in mammalian studies suggest they could be generated by similar mechanisms. Further studies are needed to determine the mechanisms behind the ripples and the correlation between slow waves and ICCs in fish.

In most fish, ripples propagated predominantly in a retrograde direction. Interestingly, this changed to a bi-directional propagation pattern after the addition of TTX, suggesting that neural input might facilitate ripples propagating in a retrograde direction. Blockade of neural activity by TTX caused an increase in the frequency of ripples in areas where they previously occurred, as well as in areas where there was no previous activity. A similar pattern is seen in mammals by suppression of neural inhibitory activity on the ICCs and/or smooth muscle (Berčík et al., 2000; Berezin et al., 1990; Smith et al., 1989).

Are there migrating motor complexes in fish?

Slow anally propagating contractions were most commonly observed in fasted shorthorn sculpin, while feeding (weekly intake of large pieces of meat) reduced the frequency. Most mammals with intermittent food intake (typically carnivores) also show distinct differences between fed and fasted (interdigestive) motility patterns, with the slowly propagating MMCs recurring at highly regular intervals in the fasted state (Buéno and Ruckebusch, 1976; Ruckebusch and Fioramonti, 1975; Ruckebusch and Buéno, 1976; Szurszewski, 1969; Wingate, 1981). In this respect, the slow anally propagating contractions in sculpin resemble MMCs; however, there are also differences that need to be considered before suggesting that these contractions could be the sculpin analogue of mammalian MMCs.

The actual velocity of MMCs depends on the species, but generally lies within the range 1–20 cm min^{-1}, decreasing with distance from the stomach (Galligan et al., 1985; Szurszewski, 1969). The velocity of slow anally propagating contractions in sculpin was in the lower end of this range, which could be partly explained by a lower body temperature compared with that of mammals. The velocity was also lower than that of spontaneously occurring contractions in the isolated proximal intestine of unfed Atlantic cod, a teleost with similar feeding habits (Karila and Holmgren, 1995). This could be due to disruption of inhibitory mechanisms (e.g. neural input), as a result of using isolated intestinal segments, which control velocity *in vivo*, or the fact that the *in vivo* preparations were under the influence of anaesthetic (Fujimiya and Inui, 2000). The mean frequency of contractions was similar in the two fish species. Likewise, many characteristics of the slow anally propagating contractions in shorthorn sculpin were similar to those of propagating contractions observed in other fish species (Gräns et al., 2009; Gräns et al., 2013; Grove and Holmgren, 1992; Holmberg et al., 2007; Holmberg et al., 2006; Holmberg et al., 2003; Holmberg et al., 2004; Karila and Holmgren, 1995).

Large variations occur between species with respect to length of the MMC period (ranging from a few minutes to several hours), as well as in the duration of phase III of mammalian MMCs (rhythmic contractions) in relation to periods of lower contractile activity (phase I–II) (Bush et al., 2000; Code and Marlett, 1975; Fleckenstein and Øigaard, 1978; Galligan et al., 1985; Ruckebusch and Fioramonti, 1975). The situation in shorthorn sculpin is closest to what occurs in mice, with short intervals between slow anally propagating contractions and less pronounced periods of quiescence or irregular contractions, which is also similar to findings from other teleosts (Bush et al., 2000; Gräns et al., 2009; Gräns et al., 2013; Holmberg et al., 2004; Karila and Holmgren, 1995).

In many species, feeding resets the MMC cycle back to phase II activity (intermittent contractions); however, MMCs often persist after food intake in continuous meal feeders (Galligan et al., 1985; Grivel and Ruckebusch, 1972; Janssens et al., 1983; Zheng et al., 2009). In birds, MMCs seem to persist regardless of feeding strategy (Clench et al., 1989). In sculpin, the presence of food in the intestine reduced the frequency of slow anally propagating contractions and the number of fish displaying this pattern. Karila and Holmgren (Karila and Holmgren, 1995) suggested that a fish analogue of MMCs was present in cod irrespective of feeding status. However, the present results suggest that a gradual change between fasted and fed patterns is more likely.

Mammalian MMCs are neurally regulated and can be abolished by blocking neural activity (Brierley et al., 2001; Spencer et al., 2000). Addition of TTX to the cod intestine reduced or abolished the spontaneous contractions, suggesting a dependence on the enteric nervous system (Karila and Holmgren, 1995). In contrast, slow anally propagating contractions in shorthorn sculpin persist in the presence of TTX, albeit with reduced velocity and amplitude (see Fig. 7). This suggests that the generation and propagation of slow anally propagating contractions is myogenic in nature but can be modulated by neural activity. However, further investigation is required to fully understand the underlying mechanisms of these contractions in sculpin.

It has been suggested that in the fasted state of many vertebrates, MMCs serve as housekeepers preventing bacteria from overgrowing and removing waste products such as undigested food and sloughed enterocytes from the gut in vertebrates (Grzesiuk et al., 2001; Kruszewska et al., 2005; Nieuwenhuijs et al., 1998).

Regardless of the underlying mechanisms, we believe slow anally propagating contractions in shorthorn sculpin may play a similar role during the interdigestive period. As fish are constantly exposed to unwanted particles including bacteria in the water, a mechanism ensuring that these particles do not accumulate in the gastrointestinal tract would be beneficial to the individual. This is especially the case in marine species, which are required to drink for osmoregulatory purposes (McCormick and Saunders, 1987).

Conclusions

We have further developed and adapted video recording and ST analysis to quantitatively describe a range of *in vivo* motility patterns that occur in the proximal intestine of a live adult fish for the first time. Using this method we showed that motility patterns present in fasted and fed fish differ, possibly reflecting differences in gastrointestinal function occurring during the two different feeding states. Furthermore, this study has shown that there are similarities and differences between fish and mammals concerning motility patterns in the proximal intestine. This method will be useful in future studies investigating *in vivo* motility patterns under different environmental conditions and will allow examination of the underlying mechanisms for the different contraction types.

MATERIALS AND METHODS

Experimental animals and treatment groups

Shorthorn sculpin (*M. scorpius*), 100–300 g (N=21), were captured off the west coast of Sweden. Fish were transported to the University of Gothenburg and kept in fibreglass tanks containing 2000 l of re-circulating, 10°C, aerated saltwater (28–30 ppt) with shelter (clay plant pots) and substrate (stones). Fish were acclimated for a minimum period of 6 weeks with a 12 h:12 h light:dark photoperiod and fed once a week with commercial white fish corresponding to ~5–15% of body mass. Prior to experimentation, fish were divided into either a ‘fed’ (fed once a week) or ‘fasted’ treatment group (no food for 3 weeks). Fish in the ‘fed’ treatment were only selected for gastrointestinal motility analysis if they had fed within the last 72 h. Research on gastric emptying time of shorthorn sculpin has shown that at 72 h post-feeding, ~20% of the meal still remains in the stomach (Seth and Axelsson, 2009). The natural feeding frequency of shorthorn sculpin is unknown; thus, we selected a fasting period of 3 weeks to ensure no food remained in the stomach or intestine. Animal care and experimental procedures were approved by the ethical committee of Gothenburg (ethical permit 368-2011) and all experiments were performed in accordance with the guidelines of the Swedish National Board for Laboratory Animals.

Experimental procedures

Individual shorthorn sculpin were initially anaesthetised in water containing 75 mg l^{-1} MS222 (ethyl-3-aminobenzoate methanesulphonic acid, $C_{10}H_{15}NO_5S$) buffered with 150 mg l^{-1} $NaHCO_3$. The length and mass of the fish were recorded prior to the fish being placed ventral side up on soft, water-saturated foam on the surgical table. To maintain anaesthesia, gills were continuously flushed with aerated water containing 50 mg l^{-1} MS222 buffered with 100 mg l^{-1} $NaHCO_3$ at 10°C (Fig. 8A).

Relative blood flow and heart rate were monitored throughout the experiment to ensure fish were sufficiently anaesthetised and healthy (Fig. 8B). This was achieved by making a small skin incision on the left side of the fish under the operculum to expose the ventral aorta and placing a 20 MHz Doppler flow crystal (Iowa Doppler products, Iowa City, IA, USA) mounted in 1.6–2.3 mm cuffs (depending on the size of the fish) on the ventral aorta adjacent to the bulbus arteriosus. The lead from the Doppler flow probe was connected to a directional-pulsed Doppler flowmeter (model 545C-4, Iowa Doppler products), which in turn was connected to a PowerLab 8/30 system (ADInstruments, Castle Hill, Australia). Data were

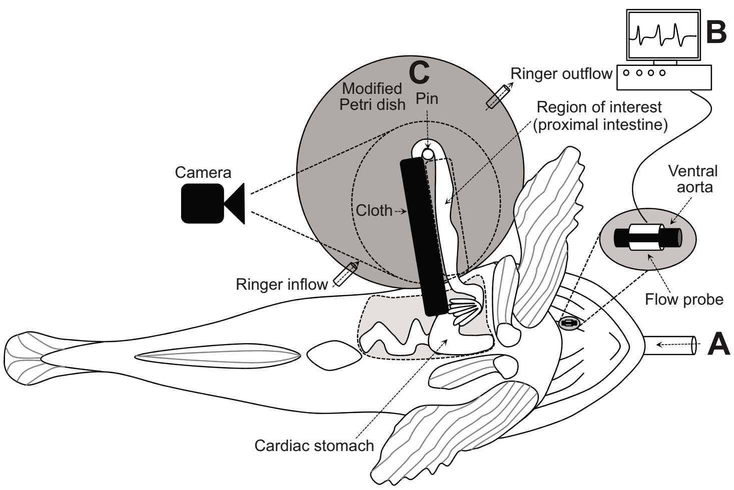

Fig. 8. Schematic drawing of the experimental setup. (A) Anaesthesia was maintained by irrigating the gills with oxygenated water containing MS222. (B) Relative blood flow and heart rate were monitored by placing a Doppler flow probe on the ventral aorta. (C) The proximal intestine was gently lifted out of the body cavity and placed on to a modified Petri dish prior to video recording.

collected on a PC using ADInstruments acquisition software Chart^{TM} 5 Pro v5.5.5, at a sampling rate of 1 kHz. 'Pre-operative' heart rate and relative blood flow of anaesthetised fish were measured for 15–20 min prior to accessing the proximal intestine. If heart rate or relative blood flow values decreased by more than 30% of 'pre-operative' values during the experimental period, then the fish was excluded.

To access the proximal intestine, a mid-ventral incision was made 10 mm anterior of the anus to 10 mm caudal of the pectoral fins. Depending on the size of the fish, ~25–55 mm of proximal intestine, beginning just after the pyloric caeca and extending approximately one-third of the total length of the intestine, was gently teased from the mid-ventral incision and placed in a modified Petri dish with black Sylgard forming the bottom layer (Fig. 8C). The proximal intestine was submerged with re-circulating shorthorn sculpin Ringer's solution (206 mmol l^{-1} NaCl, 81 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2{\cdot}2H_2O$, 1 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 1 mmol l^{-1} $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}2H_2O$, pH 7.45) bubbled with air at 10°C. Translucence of the proximal intestine made it possible to ascertain whether food contents were present or absent here in fed and fasted fish, respectively. The intestine was held in place by guiding it around a large pin positioned in the area where the intestine forms a U-bend (approximately one-third of the total length of the intestine). Care was taken not to twist, restrict or damage any blood vessels or nerves during this process. A piece of black cotton was placed over the mesentery and middle intestine, with fibre-optic lights strategically placed to ensure optimum contrast between the background and proximal intestine. The exposed peritoneal cavity was covered with chamois cloth saturated in Ringer's solution and the fish was left undisturbed for 1 h before video recording commenced.

Image recording and experimental protocol

A calibration bar (25 mm) was placed in the field of view as a spatial reference. Images were captured using a DMK31AF03 monochrome, FireWire camera (The Imaging Source, Putzbrunn, Germany). Resolution was 1024×768 pixels, corresponding to a field of view at the magnification used of 54×39 mm. Video recordings consisted of three successive 30 min long (6750 frames at 3.75 frames s^{-1}) videos. During this time, fish were left undisturbed and the temperature of both the Ringer's solution submerging the intestine and the water irrigating the gills was maintained at 10°C. During a preliminary pilot study, we observed that preparations remained viable for lengthy periods of time (>5 h) and that 90 min of video recording was sufficient to collect a sample representative of motility patterns found within individual fish.

After 90 min of video recording in the conditions mentioned above, four fasted and fed fish were exposed to TTX to examine the involvement of neural activity in the generation of intestinal motor patterns. TTX was added to the Ringer's solution to achieve a final concentration of 1 μmol l^{-1}, and fish were left undisturbed for 15 min. This was to allow the neurotoxin sufficient time to affect the proximal intestine before a 30 min video was subsequently recorded for each fish. At the end of the experimental period, fish were killed by an overdose of anaesthetic.

ST map construction

ST maps were constructed from video footage and allowed us to examine the type, speed, amplitude, direction, frequency and distribution of contractions in the intestine. ST maps display changes in diameter of the intestine over time. The first frame of each movie was imported into Volumetry G8a (author: G.W.H.) where a cropping mask was drawn over the intestine. Movies were imported and calibrated for distance and time. Distance along the proximal intestine was normalised for each fish to 100% (i.e. distance along section/total length of section). This allowed us to compare different regions of the proximal intestine in fish with varying lengths of proximal intestine. A threshold was manually selected that outlined the intestine (Fig. 9A). Background particles (such as air bubbles) were removed using a small particle filter (<1000 pixels). A region of interest was then drawn around the intestine and edge-tracking routines were employed to identify upper and lower edges of the intestine (Fig. 9B). Stub filtering was used in some experiments to remove any abrupt edge deflections caused by surface irregularities or overlap with background particles (Δslope>0.5). The distance between the upper and lower edge of the intestine was calculated for each pixel along the intestine and colour coded using a 16-bit greyscale. The smallest recorded diameter (i.e. fully contracted) was coded as black and the largest diameter (i.e. dilated or distended) as white. Each video frame produced a single row of pixels, corresponding to the diameter of each point along the preparation (Fig. 9C). Average diameter during the recorded period (30 min) was calculated and subtracted from each point along the intestine for every video frame. The resulting average background subtracted map had units of Δmm from the average diameter (Δmm; Fig. 9D). To represent the outline of the exteriorised segment of proximal intestine during the recording period, a frequency plot of the position of the intestinal edges was made, with frequency encoded as a greyscale (Fig. 9D). Pure black represents an edge remaining over the same pixel for >5 min.

Analysis of contractions from ST maps

Different types of contractions in the proximal intestine were identified using particle analysis. Firstly, a Gaussian blur was used to smooth the image (mean ± s.d. radius 15±1.5), then an aggregate of series of differential filters was used to identify the position of the peak contraction (5–15 pixels). A threshold was applied to outline contractions (Δdiameter≈0.2 mm) and then a particle analysis routine was used to create ST objects (particles separated by 4 pixels or fewer stitched together). Different criteria were then used (see below) to identify particular types of contractions.

Ripples were best isolated using a minimum size criterion followed by a linearity coefficient cutoff (r^2>0.8, Fig. 9E). The midpoint in time along the ST object was located to create a single pixel wide refined filament representing the position of the peak contraction (minimum diameter) point (Fig. 9F).

From these ST filaments, the characteristics of motion (site of initiation, distance travelled, duration, velocity and direction of propagation) as well

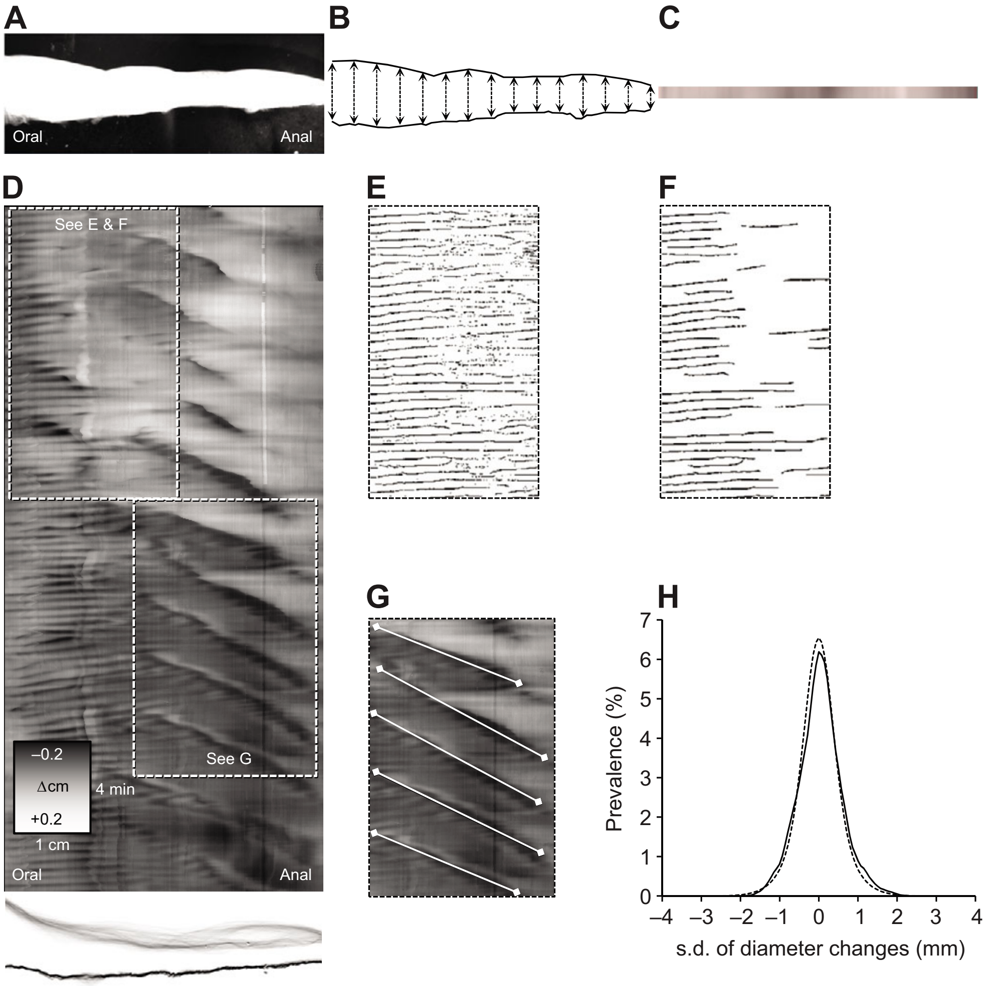

Fig. 9. Construction of a ST map of proximal intestine motor activity. Frames taken from the video recording (A) were thresholded and edge-tracking routines were employed to identify upper and lower edges of the proximal intestine to allow calculation of diameter at each pixel along the exteriorised segment of proximal intestine (B). The diameter at each point was converted to a greyscale value and plotted as a row (C) with black pixels representing minimum diameter (contraction) and white pixels representing maximum diameter (dilation). Rows of pixels, each corresponding to diameters from a single video frame were positioned underneath each other to construct a ST map (D). The overall outline of the exteriorised segment is portrayed as a greyscale-coded frequency plot of edge position (see below ST map in D). Position along intestine is on the horizontal axis, time is on the vertical axis and diameter is represented by the greyscale value (see calibration box in lower left corner). Automated identification and analysis of contractions from the ST maps were achieved using particle analysis. A series of differential filters, thresholds and criteria were used to isolated ripple-like contractions along the proximal intestine as filaments (E). Further criteria were imposed to refine the filaments and quantify the motility parameters (initiation sites, distance and time travelled, velocity, amplitude, direction) of ripple-like contractions (F). Slow anally propagating contractions were identified manually and linear lines were overlaid on the contractions to calculate motility parameters (G) similar to that for ripples. Overall contractile activity was calculated by creating a histogram of the percentage of the ST map in which the intestine was at a particular diameter value, from which s.d. of the diameter changes could be estimated (H). The ST map in D represents a recording time of 30 min.

as the number of contractions at each point along the intestine (expressed as number of contractions per minute, cpm) and the interval between contractions (‘ripple-like’ contractions constrained to between 4 and 40 s) were calculated. The amplitude of events was calculated by finding the minimum diameters in a range of ±3 s in the original ST map using the extracted refined filaments as a guide. Contraction amplitude was then normalised using the average diameter of the specific section of proximal intestine where the contraction occurred. This was done to account for the differences in average diameters between individual fish as well as different sections of the proximal intestine.

Slow anally propagating contractions were isolated manually by placing lines over the wave front edge, from which the site of initiation, distance travelled, velocity and interval between contractions were calculated from the line (Fig. 9G). Minimum diameters and amplitudes were determined in a ±5 s range for these contractions. Long cyclical periods of activity were determined manually, allowing calculation of the duration and interval between these events.

A measure of the overall contractile activity of the intestine was calculated by creating a histogram of the percentage of the ST map in which the intestine was at a particular diameter value. The resulting histograms were Gaussian in nature and a best-fit Gaussian curve was calculated to estimate the s.d. of the diameter changes (mean≈0 because of average diameter background subtraction). The larger the s.d., the greater the range of diameters experienced during the recording period (Fig. 9H).

Statistical analysis

All variables were calculated from the entire 90 min experimental period for each fasted and fed fish. However, to examine the effects of TTX on motility patterns, variables were only calculated from the 30 min experimental period directly before and after the addition of TTX. Data are presented as either mean ± s.e.m. or median depending on the shape of the distribution. Statistical analyses were performed with IBM SPSS Statistics 21 (IBM SPSS, Armonk, NY, USA). Data were assessed for normality (Shapiro–Wilk >0.05) and homogeneity of variance (Levene’s test >0.05).

If data were normally distributed, an independent *t*-test (to determine significant differences in motility patterns between fasting and fed fish) or dependent *t*-test (to determine significant effects of TTX on motility patterns) was used, whereas, if data were not normally distributed, a Mann–Whitney *U*-test was used. To determine whether feeding state (fasted or fed) was associated with slow anally propagating contractions (presence/absence), a Chi-square test for association was carried out. The Phi measure was used to determine the strength of the association between these two categorical variables. Differences where $P<0.05$ were regarded as statistically significant.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.B., G.W.H., M.A. and C.O. conceived and designed the experiments; J.B. performed the experiments and analyzed the data; J.B., G.W.H., M.A. and C.O. wrote the paper.

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