

Myotube production in fast myotomal muscle is switched-off at shorter body lengths in male than female Atlantic halibut *Hippoglossus hippoglossus* (L.) resulting in a lower final fibre number

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A sampling method is described to determine accurately the number of fast myotomal muscle fibres (N_F) in a large flatfish species, the Atlantic halibut *Hippoglossus hippoglossus*. An unusual feature of the fast myotomal muscle is the presence of internalized strips of slow muscle fibres. In fish of 1.5–3.5 kg ($n = 24$), the total cross-sectional area (A_{TC}) of fast muscle was 18% greater in the dorsal than ventral myotomal compartments ($P < 0.05$), whereas there was no significant difference between left- and right-hand sides of the body. Due to the bilateral asymmetry, muscle blocks (5 × 5 × 5 mm) were prepared to systematically sample each myotomal quadrant (dorsal, ventral, left- and right-side) and the diameters of 150 fast fibres measured per block. Smooth non-parametric probability functions were fitted to a minimum of 800 measurements of fibre diameter per quadrant ($n = 5$). There were no significant differences in the distribution of muscle fibre diameters between myotomal compartments and therefore N_F could be estimated from a single quadrant. The number of blocks required to estimate N_F with a repeatability of ±2.5% increased from six at 300 g body mass to 17 at 96.5 kg, caused by variation within and between blocks. Gompertz curves were fitted to measurements of fibre number and fork length (L_F). The estimated final fibre number was 8.96×10^5 (7.99–9.94 × 10⁵, 95% CI) for males and 1.73×10^6 (1.56–1.90 × 10⁶, 95% CI) for female fish. The estimated L_F for cessation of fibre recruitment in the fast muscle of female fish (1775 mm) was almost twice that in males (810 mm), reflecting their greater ultimate body size.

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Key words: maximum fibre number; muscle fibre recruitment; muscle fibre types; muscle growth.

INTRODUCTION

Three main skeletal muscle types are usually found in the trunk musculature of adult teleosts, classified according to colour and contraction speed as: red and slow, pink and intermediate and white and fast (Johnston *et al.*, 1977; Bone, 1978). Each fibre type expresses distinct isoforms of myosin heavy chain that

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are preceded by developmental-stage specific isoforms during the early stages of growth (Rowlerson *et al.*, 1985; Scapolo *et al.*, 1988; Crockford & Johnston, 1993; Ennion *et al.*, 1995). The axial musculature in yolk-sac Atlantic halibut *Hippoglossus hippoglossus* (L.) larvae consists of a superficial layer of embryonic slow fibres and an inner core of fast muscle fibres (Galloway *et al.*, 1999) in common with other teleost species (Van Raamsdonk *et al.*, 1978; El-Fiky *et al.*, 1987; Stickland *et al.*, 1988; Vieira & Johnston, 1992; Galloway *et al.*, 1998). Growth of the fast muscle involves the hypertrophy of the embryonic fibres until *c.* 150° days (10 mm standard length, L_S) after which additional myotubes are formed (Galloway *et al.*, 1999). Initially, fast muscle fibres are added from discrete germinal zones at the extremities of the myotomal cones (Galloway *et al.*, 1999), a growth phase known as stratified hyperplasia (Rowlerson & Vegetti, 2001). Later, new fibres are recruited on the surface of fibres formed earlier in ontogeny, producing a mosaic of fibre diameters (mosaic hyperplasia) (Weatherley *et al.*, 1979; Carpené & Verggetti, 1981; Stickland, 1983; Johnston *et al.*, 2003a). Little quantitative data, however, exists on the growth of myotomal muscle in juvenile and adult Atlantic halibut (Hagen *et al.*, 2006; Haugen *et al.*, 2006).

Atlantic halibut is the largest of all flatfish species in the Atlantic Ocean and females of 300 kg have been recorded, whereas males seldom exceed 50 kg (Moen & Svensen, 1999). In aquaculture, Atlantic halibut are rarely farmed to >4–6 kg. Metamorphosis in flatfishes is different and more extensive than in other teleosts species. Flatfishes go through a morphological transformation from being larvae to becoming a sexually immature juvenile which has most of the adult phenotypic characteristics. In Atlantic halibut, metamorphosis takes place at 10–30 mm total length (L_T) (Osse & Van den Boogaart, 1997) and results in morphological changes in most organs (Sæle *et al.*, 2004). The two most pronounced phenotypic changes during metamorphosis are the migration of the left eye to the right-hand side (right eye migration to the left occasionally happens) and the transformation from a pelagic bilaterally symmetrical larva to an asymmetric benthic juvenile. These morphological changes are under both endocrine and genetic control (Yamano *et al.*, 1991; Power *et al.*, 2001; Bao *et al.*, 2005; Tagawa & Aritaki, 2005).

The determination of fibre number (N_F) for a species is important, since flesh quality traits such as texture, are related to the number and diameters of muscle fibres (Hurling *et al.*, 1996; Johnston *et al.*, 2000a). These traits are of interest to potential Atlantic halibut breeding programmes since the final number of fast muscle fibres ($N_{F\text{final}}$) for other aquaculture species, such as Atlantic salmon *Salmo salar* L., is known to vary between families and populations (Johnston *et al.*, 2000b), has a moderate heritability (Vieira *et al.*, 2007) and correlates with growth rate (Johnston *et al.*, 2003b). For a large flatfish species, such as the Atlantic halibut, the large number of muscle fibres present, and potential differences in the distribution of fibre diameters between dorsal and ventral and left and right sides of the trunk complicate quantitative studies of muscle growth. The aim of this project was to provide information on muscle fibre growth patterns in the fast myotomal muscle of Atlantic halibut using morphometric and immunohistochemical methods. Since no previous study has attempted to estimate $N_{F\text{final}}$ in such a large fish species, the sampling methods needed to accurately estimate fibre number were investigated.

MATERIALS AND METHODS

FISH

A total of 47 Atlantic halibut were used to study muscle fibre recruitment patterns including 40 farmed fish (2 g–13 kg, 1993–2006 generations), three broodstock fish (800, 96.5 and 97.5 kg) all supplied by Mørkvedbukta Research Station (Bodø University College, Norway) and four wild fish between 6 and 50 kg (caught in Saltenfjorden outside Bodø in Norway, by local fishermen). The broodstock were originally wild caught fish that had been held in seawater tanks at ambient temperature and photoperiod (65°17' N) since 1990. In addition, 24 commercially farmed Atlantic halibut (1.5–3.5 kg) from Aga Marin AS (Dønna, Norway) were used to investigate any potential differences in fast muscle cross-sectional area between dorsal, ventral, left-hand and right-hand (Fig. 1) myotomal compartments. All the fish included in the study had normal eye migration (to the right-hand side). Fish were killed with a sharp blow to the head, except for the broodstock fish which were killed with an overdose of anaesthetic (MS-222, Argent Chemical Laboratories, Redmond, WA, U.S.A.). Twenty seven of the Atlantic halibut for the morphometric study (including the broodstock fish) and the fish from Aga Marin AS were sampled and analysed at Bodø University College. The remaining fish were transported in polystyrene boxes on ice to the Gatty Marine Laboratory (University of St Andrews, Scotland) and sampled *post-rigour* 3 days after sacrifice.

SAMPLE PREPARATION

A myotomal steak was prepared at 0.55 fork length (L_F) using a sharp knife [Fig. 1(a)]. The total cross-sectional area of fast muscle (A_{TC}) was calculated using SigmaScan Pro (v. 5.0, Systat Software, Inc., San Jose, CA, U.S.A.) from a digital photograph of the steak. One to 60 blocks (5 × 5 × 5 mm), dependent upon the body mass (M) of the fish, were prepared from the fast muscle on one or both sides of the body in order to systematically sample all areas of the myotome containing fast muscle fibres. Blocks were mounted on cork sheets and frozen in 2-methyl butane cooled to near its freezing point (−159° C) in liquid nitrogen. The blocks were wrapped in tinfoil and stored in a liquid nitrogen refrigerator until processed. The blocks were equilibrated to −18° C and 7 µm frozen sections cut on a cryostat. Serial sections were mounted on poly-L-lysine-coated slides.

HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Serial transverse sections were stained for succinic dehydrogenase (SDHase) a marker of mitochondria and aerobic capacity (Nachlas *et al.*, 1957). Sections were also stained using the S58 antibody against chicken slow myosin (obtained from the Developmental Studies Hybridoma Bank, University of Iowa, U.S.A.) (Crow & Stockdale, 1986) that has been shown to identify slow muscle fibres in a number of fish species (Devoto *et al.*, 1996; Johnston *et al.*, 2003a, 2004). Briefly, sections were fixed in acetone for 10 min and air-dried (10 min). Subsequently, sections were re-hydrated in 5% normal goat serum (v/v), 1% Triton X-100 (v/v), 1.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (w/v) for 30 min to block non-specific binding sites, followed by a three times wash in PBS (3 min) before overnight incubation (4° C) in 1:10 dilution of the S58 antibody (v/v) in 1% Triton X-100 (v/v), 1.5% in BSA in PBS (w/v). Sections were washed three times for 3 min in PBS and incubated in 1:20 anti-mouse immunoglobulin A (IgA)-biotin conjugated (v/v) secondary antibody (Sigma, St Louis, MO, U.S.A.) for 1 h at room temperature. Following 3 × 3 min washes in PBS sections were incubated in 1:20 extravidin-peroxidase (v/v) for 30 min, washed 3 × 3 min in PBS and developed with 3-amino-9-ethylcarbazole (Sigma) which produces an insoluble red end product. The reaction was stopped by washing in distilled water and the slides mounted using glycerol gelatine (Sigma).

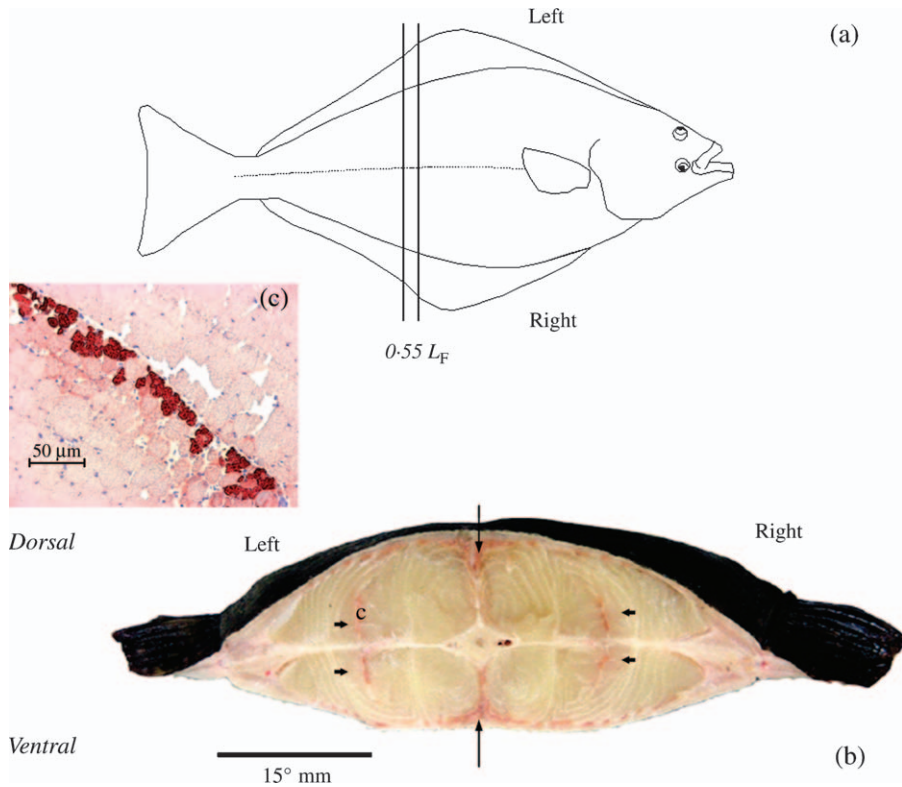


FIG. 1. (a) Muscle growth was estimated at 0.55 fork length (L_F). (b) The distribution of slow muscle is indicated by arrows (horizontal septum and internal strips). Insert (c) show S58 antibody staining of slow muscle from a 2.6 g Atlantic halibut.

MORPHOMETRIC STUDIES

Only regions of the myotome composed entirely of fast muscle fibres were sampled. For fish >1 kg, it was possible to distinguish the internal strips of slow muscle from the digital photograph of the entire myotomal cross-section [Fig. 1(b)]. In smaller fish, slow muscle fibres were distinguished by staining sections with the S58 antibody [Fig. 1(c)]. All sections were counterstained with Harris' haematoxylin (Sigma). The outlines of *c.* 150 muscle fibres per block were digitized using an Image Analysis System (Axioskop 2 mot plus; Carl Zeiss, Oberkochen, Germany) with the Axiovision 4.2 software (Carl Zeiss) and the mean A_{TC} (mm^2) calculated. For the small fish where A_{TC} was sampled in one to two blocks *c.* 1200 muscle fibres were measured. The N_F was estimated as: $N_F = N_{FC} A_{TC} A_F^{-1}$, where, N_{FC} was the cumulative number of fibres counted per block, A_{TC} is in mm^2 and A_F was the cumulative cross-sectional area of the measured fields in mm^2 . The diameters of 1200–8200 muscle fibres were measured in the dorsal, ventral, left-hand and right-hand compartments in 20 fish. For the remaining 27 fish (dependent upon size, see Fig. 2) at least 1200 fibres were measured from muscle blocks prepared from the dorsal left-hand side compartment. The colour coding of fast muscle fibres according to their size range [Fig. 3(a), (b)] was performed using SigmaScan Pro v. 5.0. (SPSS, Chicago, IL, U.S.A.).

STATISTICS

The A_{TC} in dorsal *v.* ventral, left-hand *v.* right-hand compartments in males and females were compared using a one-way ANOVA (SPSS v. 14.0.1). Non-parametric

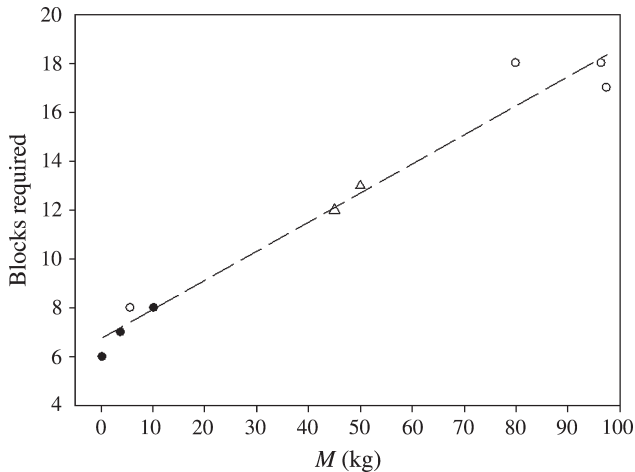


FIG. 2. The relationship between the number of blocks needed to estimate number of myotomal muscle fibres (N_F) within 2.5% accuracy and body mass (M) [Males (●), females (○) and wild fish (△, all females)]. Based on size and $N_{F\text{final}}$ the 45 kg wild fish is likely to be a female. The line presents a first order linear regression ($y = 0.12x + 6.75$; $n = 9$, $r^2 = 0.96$, $P < 0.001$).

statistical techniques were used to fit smoothed probability density functions (pdfs) to the measured muscle fibre diameters using a kernel function as described in Bowman & Azzalini (1997). The application of these methods to the analysis of muscle fibre diameters has been described in detail (Johnston *et al.*, 1999). Briefly, bootstrap techniques were used to distinguish underlying structure in the distributions from random variation (Bowman & Azzalini, 1997; Davison & Hinkley, 1997; Johnston *et al.*, 1999). The Kolmogorov–Smirnov two-sample test statistic was used to test the null hypothesis that the probability density functions of groups were equal over all diameters. To supplement this test, density curves for each treatment were compared graphically by constructing a variability band around the density estimate for the combined populations using the mean smoothing parameter. Values for the smoothing parameter, h (Bowman & Azzalini, 1997) in the compared groups were stable, ranging from 0.132 to 0.133. This provided an indication of which parts of the distribution of diameters contributed to any significant differences. The relationship between maximum fibre diameter ($D_{F\text{max}}$) and fish L_F was investigated using a linear regression analysis (SPSS).

The N_F is known to increase until a certain L_F and then reach a plateau at the final N_F . Two horizontally asymptotic growth models were initially considered based on von Bertalanffy and Gompertz curves, respectively. The curves were fitted in the non-linear curve fitting package the nlme (Pinheiro & Bates, 2000) library in R (R Development Core Team, 2007). Models were considered for each sex separately and combined. Model selection was by Akaike Information Criterion (AIC). As there was evidence of heterogeneity in the residuals, the models were fitted with a variance power function proportional to the value of the fitted value (Pinheiro & Bates, 2000). Various models were considered with different sub-sets of parameters varying by sex. The final best fit model was considered to be the Gompertz curve for each sex considered separately, with the following equation: $N_{Fi} = \alpha_j e^{[-e^{(\beta - \gamma L_{Fij})}]}$, where i and j index the i th datum within the treatment group j . α , β and γ are parameters to be estimated for each sex. α represents the asymptote, γ describes the rate at which the curve ascends and β is a constant. The L_F at which N_F reached within 1% of the estimated mean N_F determined from the asymptote of the Gompertz curve was also calculated.

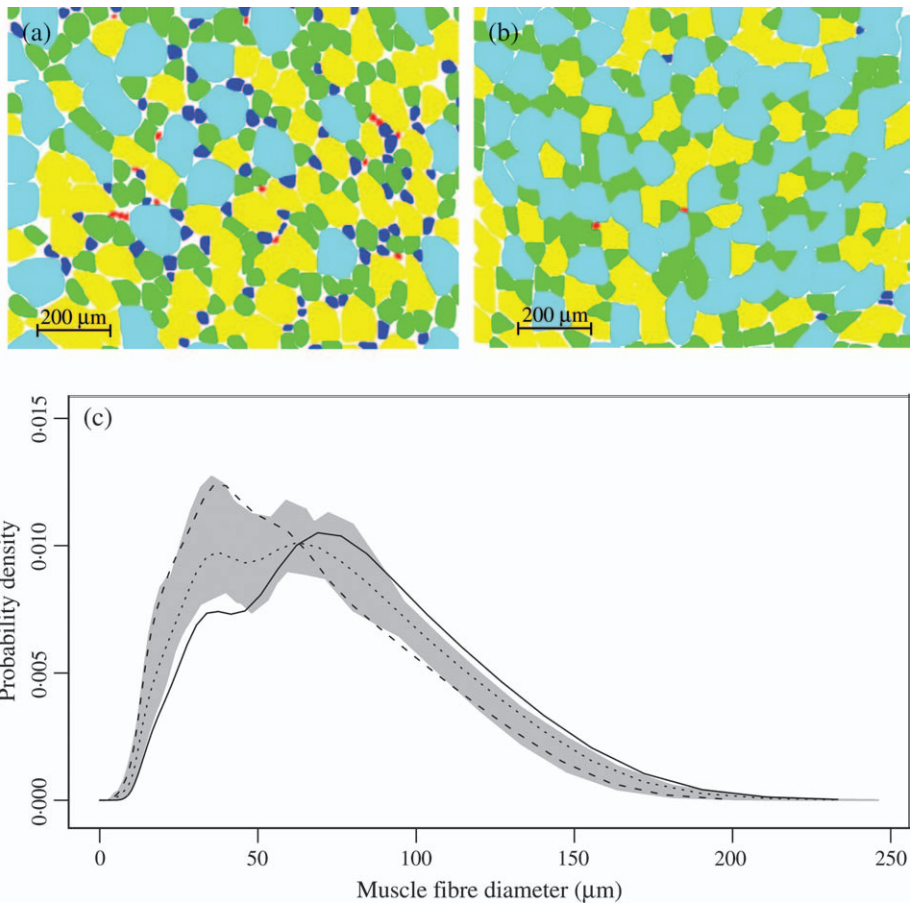


FIG. 3. Fast muscle fibres sampled from the same location from one (a) female and (b) male of identical fork length (610 mm) were colour coded and divided into size classes. Red = 0–29 μm , blue = 30–59 μm , green = 60–89 μm , yellow = 90–119 μm and aqua = >120 μm . (c) Non-parametric probability density functions were fitted to measurements of fast fibre diameter of five male (—) and five female (---) fish of c. 600 mm. The average probability density function of the combined population (····) is given.

RESULTS

MUSCLE FIBRE DIFFERENTIATION

Red muscle in Atlantic halibut was not restricted to the superficial layer under the skin, but was also found in narrow internalized strips [Fig. 1(b)]. The fibres in these internal strips of red muscle stained for slow muscle myosin [Fig. 1(c)] and had a more intensive staining for succinate dehydrogenase (SDHase) than the surrounding fast muscle fibres, which were only weakly stained.

MUSCLE FIBRE SIZE AND NUMBER

The A_{TC} of fast muscle in the dorsal myotomal compartment was 18% greater than in the ventral compartment for fish of 1.5–3.5 kg (one-way ANOVA,

$n = 24$, $P < 0.05$). No significant difference was found between A_{TC} of left-hand and right-hand compartments. There was no significant difference ($P > 0.05$) between the distribution of muscle fibre diameters either between the dorsal *v.* ventral or left-hand *v.* right-hand myotomal compartments [$n = 5$, 280–375 g; Fig. 4(a), (b)]. Similar results were obtained for the three brood-stock fish.

The number of blocks required to estimate fibre number with a repeatability of $\pm 2.5\%$ increased from one in fish of 2.6 g to six in a fish of 300 g and 17 in a fish of 96.5 kg [Figs 2 and 4 (c), (d)]. On the other hand, for the smallest size classes of fish where the A_{TC} was sampled in one to two blocks, 800 muscle fibres was found necessary to achieve a reliable estimate.

Smooth distributions were fitted to 1200 measurements of fibre diameter per fish using a kernel function and the corresponding probability pdfs were

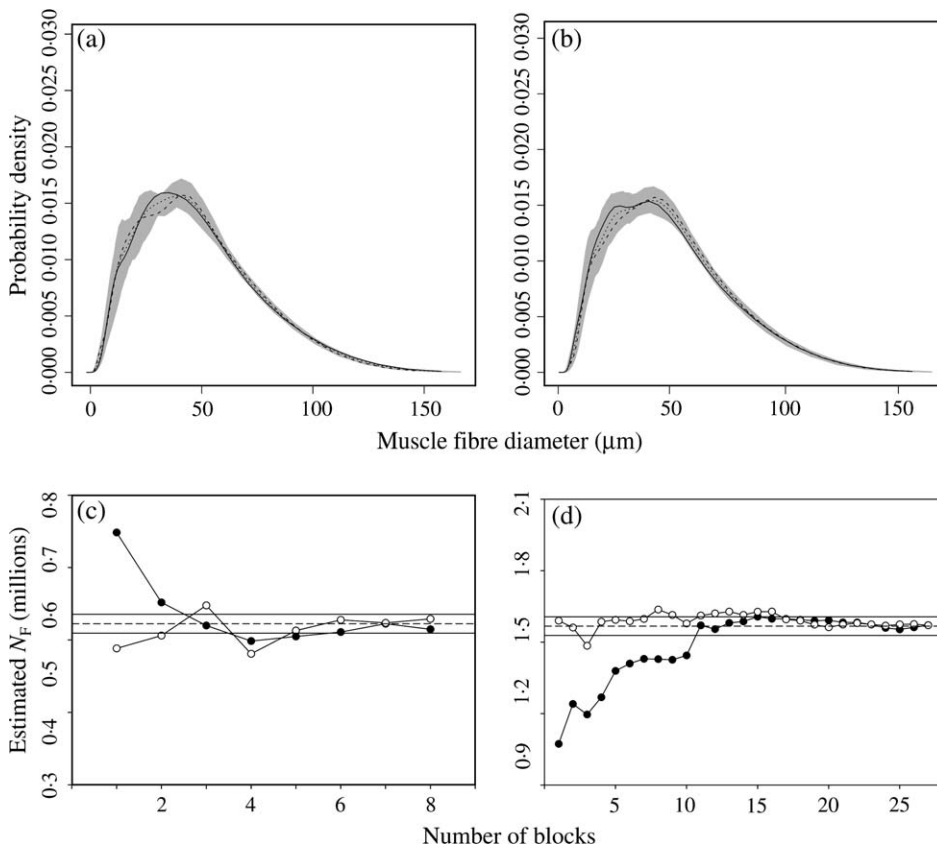


FIG. 4. The distribution of fast muscle fibre diameters in (a) dorsal (.....) and ventral (—) and (b) left-hand (.....) and right-hand (—) myotomal compartments ($P > 0.05$) (....., the mean value). The number of blocks (c. 150 fibres per block) needed to estimate the $N_{F\text{final}}$ in a (c) 0.3 kg juvenile (>6 blocks) and (d) 96 kg adult Atlantic halibut (>17 blocks) with a repeatability of $\pm 2.5\%$ is given (---, the muscle N_F estimated using all measured blocks) in dorsal (○) and ventral (●) compartments, respectively.

plotted. A selection of the data from seven out of 47 Atlantic halibut is shown in Fig. 5(a). As the fish grew there was a right-hand shift in the distribution of muscle fibre diameter. There was a linear relationship between the $D_{F_{\max}}$ (calculated from the 97th percentile of diameters) and L_F [Fig. 5(b)] ($n = 47$, $r^2 = 0.89$, $P < 0.001$). In the wild caught fish of 45 kg $D_{F_{\max}}$ was 360 μm , while $D_{F_{\max}}$ for the broodstock varied from 280 to 330 μm [Fig. 5(b)].

Fast muscle fibres sampled from the same location (dorsal left side close to the horizontal septum) of one male and one female of identical size (610 mm) was given colour codes based on the difference in fibre size range to illustrate the difference between sexes [Fig. 3(a), (b)]. The colouration for the muscle fibres shows that male fish has very few small fibres compared to female fish [Fig. 3(a), (b)]. Comparison of the distribution of fast fibre diameters in five males and five females of similar size (c. 600 mm) showed that males had fewer muscle fibres in the smaller size classes (10–50 μm), but a higher percentage of large diameter fibres (90–200 μm) than females [Fig. 3(c)] (Kolmogorov–Smirnov test, $P < 0.05$).

In order to obtain estimates of the $N_{F_{\text{final}}}$ and the L_F at which fibre recruitment ceased, the data on N_F was fitted to separate Gompertz growth models for each sex. The $N_{F_{\text{final}}}$ was set to 1% of the asymptotic value of the curve to obtain a model output that was broadly consistent with the information collected on muscle fibre diameters (Fig. 6). The estimated final $N_{F_{\text{final}}}$ was 8.96×10^5 ($7.99\text{--}9.94 \times 10^5$, 95% CI) for males and 1.73×10^6 ($1.56\text{--}1.90 \times 10^6$, 95% CI) for female fish. The estimated L_F for cessation of fibre recruitment

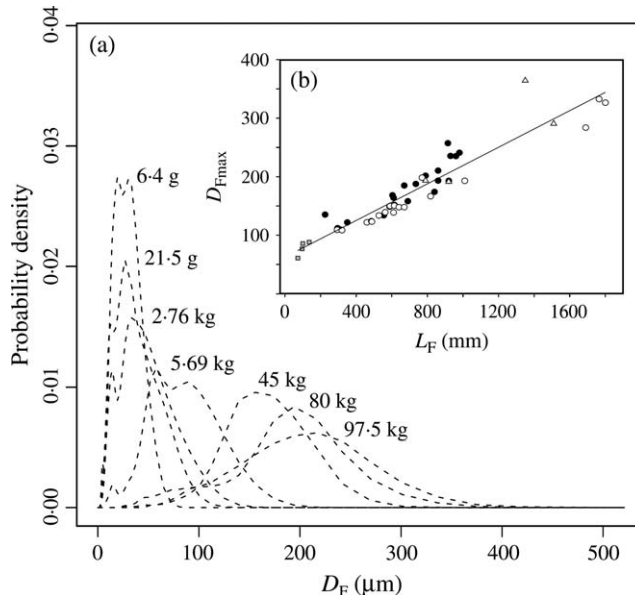


FIG. 5. (a) The probability density functions of fast muscle fibre diameter (D_F) for seven of 47 Atlantic halibut investigated. (b) A linear regression was fitted to the maximum fibre diameter ($D_{F_{\max}}$) calculated from the 97th percentile of the distribution and fork length (L_F) ($y = 1.56x + 62.58$; $n = 47$, $r^2 = 0.89$, $P < 0.001$) for males (●), females (○), sex unknown (□) and wild fish (△, all females). Based on L_F and $N_{F_{\text{final}}}$ the 45 kg (1350 mm) fish is likely to be a female.

in the fast muscle of female fish (1775 mm) was almost twice that in males (810 mm).

DISCUSSION

In the present study, it was shown that the distribution of fast muscle fibre diameters in Atlantic halibut is similar between myotomal compartments [Fig. 4(a), (b)], such that N_F can be estimated from measurements of any quartile. Furthermore, the number of blocks (*c.* 150 fibres per block) required to sample N_F with a repeatability of $\pm 2.5\%$ has been determined for fish up to 96.5 kg. In such large fish, it is necessary to quantify the cross-sectional areas of >2500 individual fibres distributed between 17 muscle blocks ($5 \times 5 \times 5$ mm) to obtain a reliable estimate of N_F . It has recently been suggested that discrete growth zones are present in the myotomes of adult Atlantic halibut (Haugen, 2006), however, no evidence for this was found in the present study.

Previous studies with 1–2 kg Atlantic halibut had shown that females had a higher N_F than males (Hagen *et al.*, 2006). In the present study, it was established that $N_{F\text{final}}$ was *c.* 1.9 fold higher in female (1.73×10^6) than male (8.96×10^5) fish reflecting the greater ultimate size of females. Furthermore, this increase in $N_{F\text{final}}$ of fast muscle was achieved by delaying the switching off of myotube production to longer body lengths in female (*c.* 1775 mm L_F) than in male (*c.* 810 mm L_F) fish (Fig. 6). These estimates of the L_F at which fibre recruitment stops should be regarded with some caution due to the relatively small number of fish studied. Male fish matured at a slightly shorter L_F (Jákupsstovu & Haug, 1988) than the L_F at which recruitment stops (Hagen *et al.*, 2006). It is interesting to note that females do not become sexually matured until 1100–1150 mm (Jákupsstovu & Haug, 1988), such that muscle fibre recruitment ceases at a larger L_F than sexual maturity which is similar to the situation in males. The literature regarding maximum L_F of female and male Atlantic halibut is not consistent, and data on maximum L_F in males is not very well established. Assuming that the maximum L_F is

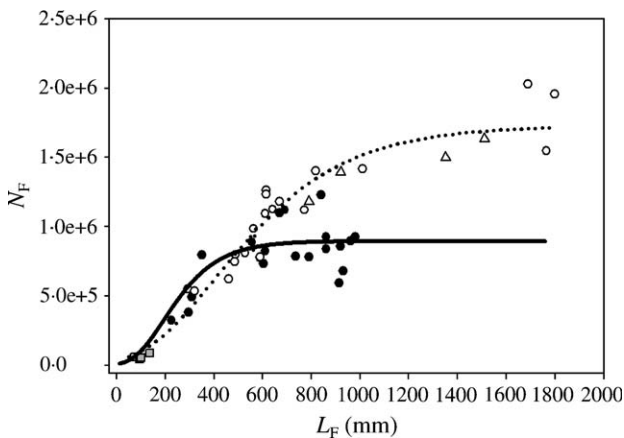


FIG. 6. Gompertz curves were fitted separately to measurements of muscle (N_F) and (L_F) for male (—; ●) and female (---; ○) [sex unknown (□) and wild fish (Δ, all females)].

c. 1900 (Bowering, 1986) and 3000 mm (Moen & Svensen, 1999) for males and female, respectively, the recruitment of fast muscle fibres stopped at c. 43 and 59% of the ultimate L_F . In 10 freshwater species, fast muscle hyperplasia was found to cease at 44% of the ultimate L_F (Weatherley *et al.*, 1988).

Evidence for a similar sexual dimorphism in fast muscle fibre recruitment patterns has been reported for the Argentine hake (*Merluccius hubbsi* Marini) in which females also reach a larger body size than males (Calvo, 1989). In mammals, males are often larger than females and this sexual dimorphism is associated with greater muscle mass (Shahin, 1995). It has been suggested that this sexual dimorphism is attributable to differences in androgens (Bardin & Catterall, 1981), insulin-like growth factor I (Liu *et al.*, 2000), growth hormone (Udy *et al.*, 1997) and the processed form of myostatin (McMahon *et al.*, 2003). The physiological mechanisms regulating differences in muscle fibre recruitment between male and female fishes showing a sexual dimorphism in body size are unknown.

At all stages of ontogeny muscle growth involves an increase in the length and diameter of muscle fibres $< D_{Fmax}$. The 45 kg wild Atlantic halibut was identified as an outlier in [Fig. 5(b)], having the largest D_{Fmax} , considerable larger than the largest of the broodstocks. This fish was in an exceptional good condition having the largest condition factor of all halibut investigated and a A_{TC} almost as large as the 80 kg broodstock Atlantic halibut (pers. obs.). The maximum diameter is probably limited by diffusional constraints and the need to avoid an anoxic core in the centre of the fibre (Egginton *et al.*, 2002). Resting and maximum mass-specific metabolism scales with $M^{-0.25}$ (Schmidt-Nielsen, 1984), and thus the maximum permissible diameter is expected to increase with L_F [Fig. 5(b)] as diffusional constraints are relaxed (Johnston *et al.*, 2003a). The D_{Fmax} was a linear function of L_F in Atlantic halibut as has been reported for sub-Antarctic and Antarctic notothenioids living at cold temperatures (Johnston *et al.*, 2003a). In contrast, in some species *e.g.* Atlantic salmon *S. salar* L., the maximum diameter reaches a limiting value and then becomes independent of L_F (Johnston *et al.*, 2003b). The relationship between D_{Fmax} and L_F was the same for male and female Atlantic halibut suggesting that similar diffusional constraints operate in the muscle fibres of both sexes. In contrast, c. 600 mm L_F males have a higher percentage of muscle fibres with diameters in the largest size classes less than D_{Fmax} than females [Fig. 3(a–c)]. It has previously been proposed an optimum N_{Ffinal} hypothesis to explain intra- and inter-specific differences in muscle fibre size (Johnston *et al.*, 2003b, 2005, 2006). Theoretically, as the surface-to-volume ratio of muscle sarcolemma decreases with an increase in fibre diameter, so do the passive leak of ions across the muscle membrane and hence the cost of ionic homeostasis involving ATP-dependent pumps of various kinds. Thus, routine maintenance costs are minimized if the fibres are as large as possible without incurring diffusional constraints, which sets the N_{Ffinal} at some optimal level. In the case of Atlantic halibut, growth to a large body size by females presumably brings benefits in terms of fecundity. In order for female fish to achieve a larger ultimate body size than males which mature earlier, a higher N_{Ffinal} is required, and this may bring a penalty in terms of a higher routine maintenance cost due to the associated smaller average fibre diameter, in particular for immature individuals.

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