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MUSCLE FIBRE TYPES OF FISHES;
STRUCTURAL AND FUNCTIONAL SPECIALIZATION

CENTRALE LANDBOUWCATALOGUS
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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
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DER
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WAGENINGEN

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STELLINGEN

1. Bij spiervezels kan een groot aantal combinaties van verschillende functionele eigenschappen voorkomen. Een bruikbare indeling van deze spiervezels in typen vereist dat men één eigenschap als hoofdcriterium kiest, indeelt op kenmerken die hiermee verband houden en vervolgens op grond van andere eigenschappen een steeds verder gaande opsplitsing maakt.

2. Voor het onderscheiden van snelle en langzame spiervezels is de relatieve lengte van het contact tussen T-systeem en sarcoplasmatisch reticulum een beter structuurkenmerk dan het relatieve volume van het sarcoplasmatisch reticulum.
   Dit proefschrift.

3. De positie van het T-systeem hangt doorgaans samen met de lengte van de actinefilamenten en daarmee indirect met de sarcomeer lengte-spannings relatie; slechts in enkele gevallen is deze positie gerelateerd aan de mate van contact tussen het T-systeem en het sarcoplasmatisch reticulum.
   Dit proefschrift.

4. Het monopoliseren van de term 'slow fibre' voor de tonische spiervezel zoals die bij amfibiën is beschreven (Morgan en Proske) en het definiëren van 'slow-twitch fibres' als vezels die een actiepotentiaal voortgeleiden (Vrbová et al.) laat weinig ruimte open voor een benaming voor langzame spiervezels van vissen.
   Morgan, D.L., Proske, U. 1984 Physiol. Rev. 64: 103-169
   Vrbová, G., Gordon, T., Jones, R. 1978 Nerve-muscle interaction, Chapman and Hall, London

5. De algemeen gangbare opvatting dat bij naast elkaar liggende sarcomeren gelijknamige banden precies op een rij liggen, zodat deze banden over de hele spiervezel op dezelfde hoogte liggen. (Ham, Junqueira en Carneiro; Eckert en Randall) is onjuist.
   Eckert, R., Randall, D. 1983 Animal Physiology, W.H. Freeman and Company, San Francisco. p. 344.
   Ham, A.W. 1974 Histology, J.B. Lippincott Company, Philadelphia, Toronto. 7e ed. p. 528.
   Junqueira, L.C. Carneiro, J. 1981. Functionele histologie, Bunge, Utrecht. p. 240.
   Zie ook: Banks, R.W. 1981 J. Anat.137: 414-415 en Rüdel, R., Thaer, A. 1981 J. Physiol. London. 318: 28P.

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6. Het is waarschijnlijk dat in N lijn, die vooral bij lange sarcomeren wordt waargenomen, de actinefilamenten onderling verbonden zijn door eiwit structuren die een rol spelen bij de overgang van het tetragonaal actinefilament patroon bij de Z lijn naar het hexagonaal patroon in de A band.

   Wang, K., 1984 in: Pollack, G.H. en Sugi, H. (eds) Contractile mechanisms in muscle. Advances in Experimental medicine and biology 170: 285-303, Plenum Press, New York, Lomdon.

7. De waarneming van Wigmore en Stickland dat grotere varkensfoetussen ongeveer evenveel primaire maar meer secundaire embryonale spiervezels hebben dan hun kleinere toomgenoten geeft geen informatie over post-natale verschillen in vezeltype tussen toomgenoten.

   Wigmore, P.M.C., Stickland, N.C. 1983 J. Anat. 137: 235-247.

8. In tegenstelling tot de hypothese van Stickland en Goldspink is het spiervezelaantal in de m.flexor digiti V brevis van het varken geen interessant selectiekenmerk voor het verhogen van de vleesproduktie bij varkens.

   Kanis, E., Akster, H.A., Bouman, T.G.H. 1983 CEC Workshop: In vivo measurement of body composition in meat animals. Langford, Bristol.
   Stickland, N.C., Goldspink, G. 1975 Animal. Prod. 21: 93-96.

9. Het samenvatten van gecompliceerde gegevens in overzichtelijke schema's werkt vaak zeer verhullend. Schema's dienen dan ook met argwaan te worden bekeken.

10. Als men in een eigen publicatie door anderen gepubliceerde onzin wil bestrijden, moet men er rekening mee houden dat dit de impactfactor van die onzin verhoogt.

11. Het feit dat deskundigen aan wie de beoordeling over plaatsing van manuscripten in tijdschriften wordt toevertrouwd, vaak niet gerechtigd zijn zitting te nemen in een promotiecommissie, is kenmerkend voor het traditionele karakter van promoties.

Proefschrift van H.A. Akster
Muscle fibre types of fishes;
structural and functional specialization
Wageningen, 5 oktober 1984

INHOUD / CONTENTS

ALGEMENE INLEIDING

1. VRAAGSTELLING

Het spierstelsel is het omvangrijkste orgaansysteem van het dierlijk, en menselijk, lichaam; meer dan veertig procent van het lichaamsgewicht bestaat uit spier. Vanuit landbouwkundig gezichtspunt is het het meest interessante orgaanstelsel; vee- en visteelt zijn in sterke mate gericht op het efficient produceren van spierweefsel.
Het verschil tussen rood en wit vlees is al zeer lang bekend. De eerste die dergelijke kleurverschillen, bij spieren van het konijn en de rog, in verband bracht met verschillen in spierfunctie was Ranvier (1873). Het karakteriseren van verschillende typen spiervezels en het nagaan van hun functionele betekenis voor het dier, is de hoofdlijn van het in dit proefschrift beschreven onderzoek.

Spieren zijn betrokken bij het uitvoeren van zeer uiteenlopende bewegingen. Dit geldt zeker voor spieren van vissen. Eenzelfde spier kan zowel betrokken zijn bij voedselopname, vaak prooivangst, als bij ademhaling. Prooivangst is een proces dat zich zeer snel (binnen 20 msec, Muller, 1983) afspeelt en waarbij grote versnellingen (meer dan tien maal de versnelling van de zwaartekracht, van Leeuwen, 1983) worden uitgeoefend, terwijl ademhaling een langzame,maar continu ritmisch herhaalde beweging is. Het uitvoeren van zulke geheel verschillende bewegingen door dezelfde spieren is aanleiding tot het stellen van de vraag:
"Hoe voldoet een spier aan de, deels tegenstrijdige, eisen die er voor het uitvoeren van verschillende bewegingen aan gesteld worden".
De grote variatie in aquatisch milieu, en in daaraan aangepaste typen vissen, brengt veel verschillende manieren van voedselopname met zich mee. Het is te verwachten dat deze verschillen tot uiting komen in verschillen in bouw van de spieren van deze vissen.
Boddeke, Slijper en van der Stelt beschreven in 1959 een met het zwemgedrag samenhangend verschil in samenstelling van de rompspieren bij diverse groepen vissen. Vissen die een groot gedeelte van hun tijd zwemmen (stayers) bleken relatief meer dunne, rode spiervezels te hebben dan vissen die slechts

gedurende korte tijd een prooi achtervolgen of zelf wegvluchten (sprinters). Bone (1966) en Rayner en Keenan (1967) toonden met behulp van electromyografie bij de hondshaai *Scyliorhinus canicula L.* en de tonijn *Katsuwonus pelamis L.* aan dat het rode deel van de rompspieren inderdaad gebruikt wordt tijdens langzaam en langdurig zwemmen. Het grotere, witte deel wordt pas actief bij hogere zwemsnelheden.

In aansluiting op deze gegevens is in dit proefschrift de hierboven gestelde vraag aangepakt door onderzoek te verrichten naar het voorkomen van verschillende typen spiervezels in de spieren van vissen. Vervolgens werd nagegaan wat de relatie is tussen de histochemische, structurele en contractiele eigenschappen van deze typen en welke betekenis deze eigenschappen hebben voor het functioneren van dit vezeltype in de vis. Dit onderzoek kan dan ook worden omschreven als functionele morfologie op cellulair niveau.

Inzicht in de functionele betekenis en kennis van de histochemische en structurele kenmerken van deze typen is noodzakelijk om vragen naar de ontwikkeling, differentiatie en groei van spiervezels en spiervezeltypen te kunnen beantwoorden. Over differentiatie van spiervezels en spiervezeltypen is reeds onderzoek verricht (zie voor een overzicht van Raamsdonk, 1982), maar er zijn nog vele onbeantwoorde vragen. Voor de post-larvale groei en de veranderingen in spiervezelsamenstelling tijdens de groei (zie Stickland, 1983) geldt dit in nog sterkere mate.

Ter verduidelijking wordt hieronder eerst de algemene bouw en werking van de dwarsgestreepte spiervezels van gewervelde dieren kort aangeduid. Daarna worden de gronden waarop deze spiervezels in typen worden onderverdeeld besproken.

2. BOUW EN WERKING VAN SPIEREN

2.1. Bouw en contractie van spiervezels

Dwarsgestreepte spieren zijn opgebouwd uit spiervezels en uit voedende, stevigheid en samenhang verlenende en besturende elementen: bloedvaten, bindweefsel, zenuwen en zintuigorgaantjes. Het bindweefsel zet zich voort in de pees en brengt de door de spiervezels uitgeoefende kracht over op beenelementen. De levering van kracht (omzetting van chemische energie in mechanische energie) door de spiervezels vindt plaats in spierfibrillen of myofibrillen, langgerekte structuren waarin zones met dunne eiwitketens, actine filamenten, en zones met dikkere eiwitketens, myosine-filamenten, elkaar afwisselen. De myosine- en de actine filamenten overlappen elkaar gedeeltelijk (zie fig. 1 en fig. 2). Door deze rangschikking ontstaat een patroon van lichte en donkere banden; de dwarsstreping waaraan deze spieren hun naam ontlenen. De namen van de banden

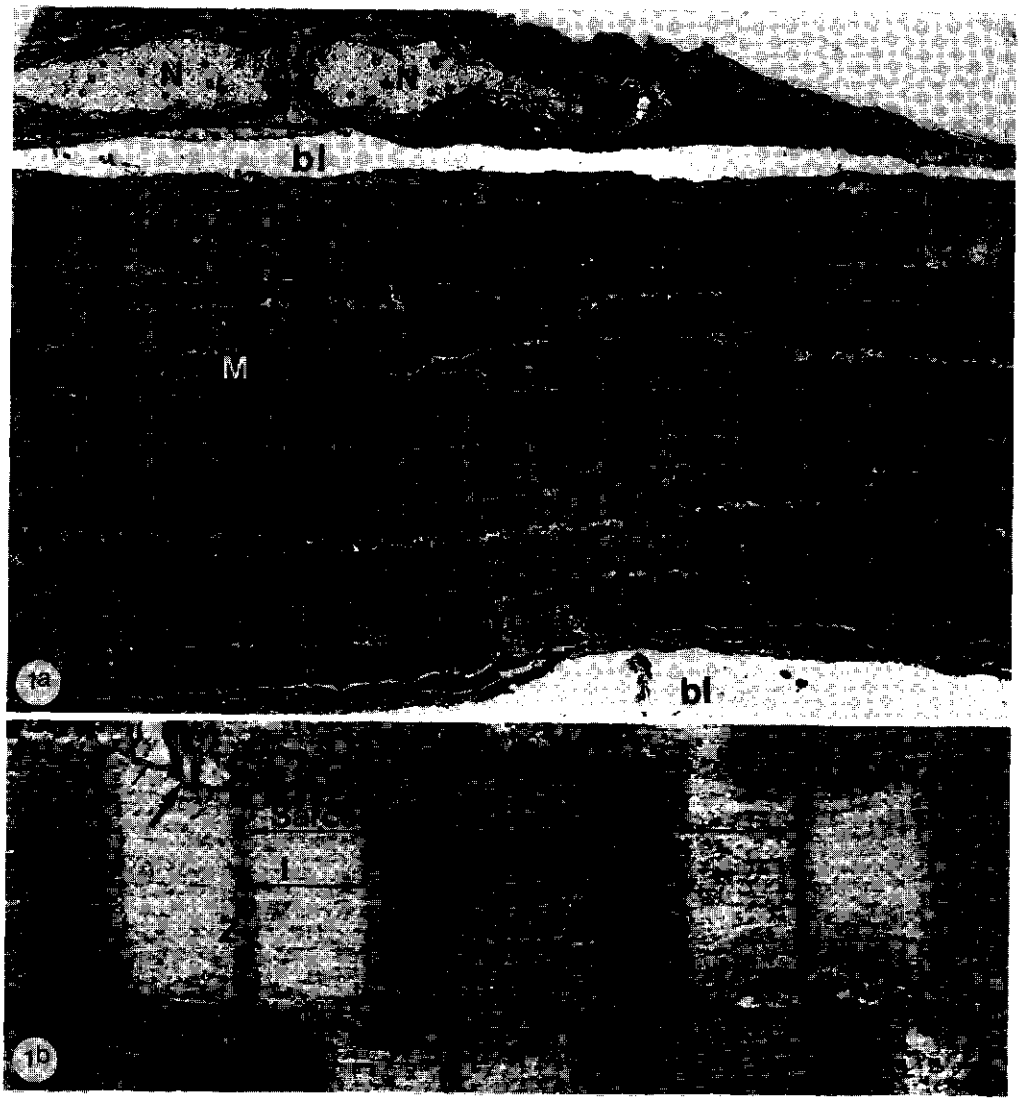

Fig. 1a. Overlangse doorsnede van een dwarsgestreepte spiervezel met daaromheen bloedvaatjes (bl) en zenuwvezels (N). Behalve de dwarsstreping is ook een, door de myofibrillen veroorzaakte, lengtestreping in de vezel te zien. Deze, rode vezel bevat vrij veel mitochondria (M). Vergroting 4000 x.

Fig. 1b. Deel van een myofibril. De verschillende banden van een sarcomeer zijn aangegeven (zie ook fig. 2). De A band bevat myosine filamenten, deze zijn in het midden, in de M lijn, onderling verbonden. De I band en het deel van de A band buiten de H band bevatten actine filamenten. De actine filamenten zijn onderling verbonden in de Z lijn.
dunne pijlen: doorsneden door het sarcoplasmatisch reticulum; dikke pijl: T systeem. Vergroting 28.000 x.

zijn aangegeven in fig. 1b. Het deel van een myofibril dat tussen twee Z lijnen inligt, heet een sarcomeer.

De nu algemeen gangbare theorie over spiercontractie is de sliding filament- cross bridge theorie van A.F. Huxley and H.E. Huxley (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). Deze theorie gaat uit van het sarcomeer als contractiele eenheid. Als de spier contraheert, schuiven de actine filamenten verder naar binnen tussen de myosine filamenten, zodat het sarcomeer zich verkort. De I band en de H band worden hierbij korter. Dit in elkaar schuiven gebeurt doordat beweeglijke uitsteeksels, de myosinekoppen, die op regelmatige afstand op de myosine filamenten voorkomen, verbindingen aangaan met de actine filamenten en deze verder naar het midden van het sarcomeer trekken. Deze brugvorming is een cyclisch proces waarbij ATP wordt verbruikt. De herhaling van de cyclus kan op twee manieren doorbroken worden: door het blokkeren van de bindingsplaats op het actine-filament of door het opraken van ATP. Als de spier in rust is, zijn de bindingsplaatsen op het actine-filament geblokkeerd.

Iedere myofibril is omgeven door het sarcoplasmatisch reticulum, een uit membranen opgebouwd systeem van dunne buizen en verwijde blazen, die met elkaar in verbinding staan (fig. 2).

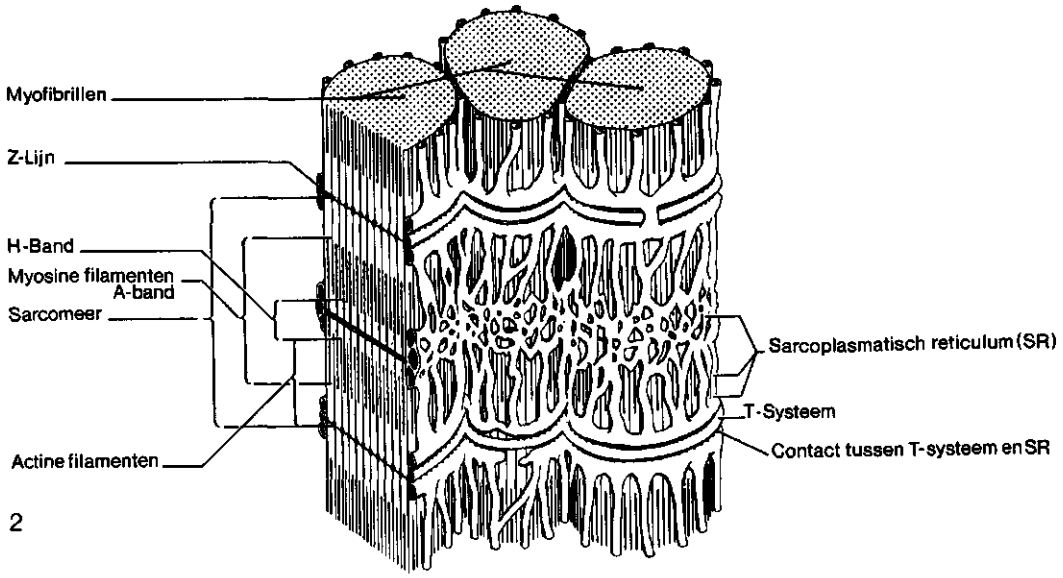

Fig. 2. Schematische weergave van myofibrillen met de hun omgevende membraansystemen (sarcoplasmatisch reticulum en T systeem).

Per sarcomeer wordt het sarcoplasmatisch reticulum een of twee keer onderbroken door het T-systeem; dit bestaat uit buisvormige instulpingen van de vezelmembraan die voornamelijk dwars op de richting van de myofibrillen verlopen. Het sarcoplasmatisch reticulum heeft aan weerszijden van het T-systeem verwijdingen, de eindblazen, die contact maken met het T-systeem.

2.2. Activatie van een spiervezel

Bij activatie van de spier door een zenuwimpuls, wordt de membraan van de spiervezel gedepolariseerd. Deze depolarisatie zet zich voort over de membraan van het T systeem naar het inwendige van de spiervezel. Onder invloed van deze depolarisatie komt,op nog onopgehelderde wijze,calcium vrij uit het sarcoplasmatisch reticulum. Dit calcium bindt zich aan een van de componenten (troponine C) van de troponine-tropomyosine complexen die zich op de actine filamenten bevinden (Ebashi and Endo, 1968). Hiermee wordt de blokkering voor de vorming van bruggen tussen de myosine- en actine filamenten opgeheven. In het sarcoplasma, vooral in dat van vissen, bevindt zich een eiwit, het parvalbumine, dat ook calcium bindt (Gerday and Gilles, 1976; Hamoir et al., 1981; Gerday, 1982). Parvalbumine heeft een hogere affiniteit voor calcium dan troponine. De binding aan parvalbumine verloopt echter langzamer dan die aan troponine,omdat de bindingsplaatsen op het parvalbumine aanvankelijk nog bezet zijn door magnesium (Gilles et al.,1983)..Het calcium bindt zich dan ook eerst aan het troponine, maar wordt al vrij snel overgenomen door het parvalbumine. Het sarcoplasmatisch reticulum heeft weer een grotere affiniteit tot calcium dan het parvalbumine, zodat het calcium uiteindelijk in het sacroplasmatisch reticulum wordt terug gepompt. Dit is een proces waarbij ATP wordt verbruikt. Als er geen calcium meer aan het troponine gebonden is, worden de bindingsplaatsen voor de myosinekoppen weer geblokkeerd en stopt de contractie. Om de spiercontractie te laten voortduren, is dan ook het voortdurend toedienen van nieuwe impulsen, die tot het vrijkomen van calcium leiden,vereist.

Er bestaan diverse typen spiervezels die verschillen in de snelheid waarmee het hierboven beschreven proces zich voltrekt. Dit houdt onder andere verband met verschillen in:

- uitgebreidheid van het T systeem (Nag,1972; Korneliussen en Nicolaysen, 1975; Eisenberg en Kuda,1976).
- structuur en eigenschappen van het regulerende eiwit troponine (Perry et al., 1979).
- structuur en eigenschappen van de contractiele eiwitten actine en myosine (Syrovy et al., 1970; Lowey en Risby, 1971; Focant et al., 1976; Pool et al., 1976).
- activiteit van het myosine ATPase (Bárány, 1967).
- het voorkomen van verschillende typen en verschillende concentraties parvalbumine (Hamoir et al., 1972, 1981; Gerday, 1982).
- de uitgebreidheid van het sarcoplasmatisch reticulum (Nag, 1972; Eisenberg en Kuda, 1976; van Winkle en Schwarz, 1978; Somlyo et al., 1979).
- de snelheid waarmee het sarcoplasmatisch reticulum calcium bindt (Sreter, 1969; van Winkle et al.,1978; McArdle and Johnston, 1981).

Van deze verschillen kan gebruik worden gemaakt voor het karakteriseren van typen spiervezels.

2.3. Typen spiercontractie (naar Wilkie, 1976 en Close, 1972)

Een spier in rust heeft een zekere elasticiteit. Deze wordt voornamelijk veroorzaakt door het in de spier aanwezige bindweefsel. Een deel van deze elastische elementen ligt parallel aan de contractiele elementen, de sarcomeren; een deel is ermee in serie gerangschikt. Als een spier kracht uitoefent op een last, zal er een vertraging optreden tussen de krachtsontwikkeling in de sarcomeren en de waarneembare spierwerking omdat eerst de elastische elementen gespannen worden.

Als een spier met een *twitch* reageert op een enkele prikkel, duurt de periode waarin de bruggen tussen myosine en actine actief zijn slechts kort; te kort om de elastische elementen zover te strekken dat de spanning die door de contractiele elementen wordt geproduceerd ook aan de uiteinden van de spier merkbaar is. Als de periode waarin de bruggen actief zijn door een volgende

prikkel wordt verlengd, of hernieuwd voordat de in de elastische elementen opgebouwde spanning geheel is verdwenen, gaat het strekken van die elementen door en wordt een grotere kracht door de spier uitgeoefend. Als de impulsen elkaar zo snel opvolgen dat de spier steeds spanning blijft behouden,is een *tetanus* ontstaan. Als de impulsfrequentie zo hoog is, dat de bruggen tussen myosine en actine ononderbroken actief zijn, kan de spier zijn maximale spanning bereiken. De spier vertoont dan een *maximale tetanus*.

Krachtsuitoefening door spieren wordt op twee verschillende manieren gemeten: *isometrisch*, waarbij de lengte constant gehouden wordt en *isotonisch*, waarbij men de spier tegen een constante belasting laat verkorten. Bij natuurlijke bewegingen varieert meestal zowel de lengte als de belasting van de spier.

Bij meting van de isometrisch uitgeoefende kracht treedt, hoewel de lengte van de spier constant gehouden wordt, toch enige verkorting van de contractiele elementen op. Hierdoor worden de elastische elementen gestrekt en wordt kracht uitgeoefend op de gebruikte transducer. De kracht die tijdens een isometrische contractie wordt gemeten, is afhankelijk van de lengte waarop de spier wordt gefixeerd. De lengte-spanningscurve van een spier is afhankelijk van de bouw van de sarcomeren (zie voor de invloed van de lengte van de actine filamenten hierop de hoofdstukken II en VII) en van het gedrag van de elastische elementen.

De snelheid van een isotonische contractie is afhankelijk van de belasting. Als de belasting te zwaar is, zal de spier niet verkorten en bij een supramaximale last kunnen de bruggen tussen myosine en actine de last niet houden; ze zullen slippen en de spier wordt uitgerekt. De maximale snelheid die de spier kan bereiken, wordt bereikt als de belasting gelijk is aan nul. Deze maximum snelheid is afhankelijk van de intrinsieke snelheid, dit is de snelheid waarmee de sarcomeren verkorten, en van het aantal sarcomeren in serie. De intrinsieke snelheid is gerelateerd aan de activiteit van het myosine ATPase; hoe hoger deze activiteit is, des te sneller contraheert de spier (Bárány, 1967). Daar de intrinsieke snelheid van een spier(vezel) lastig te bepalen is, wordt voor het vergelijken van snelheden van spieren vaak de tijd die verstrijkt tussen de stimulus en het bereiken van de maximale kracht (time to peak tension) van een isometrische twitch genomen. Deze methode is in de hoofdstukken IV en VI gevolgd.

3. INDELING VAN SPIERVEZELTYPEN

3.1. Tonische en fasische vezels

De eerste onderverdeling, die algemeen aangebracht wordt bij dwarsgestreepte spiervezels van gewervelde dieren, is die in snelle, fasische of twitch vezels en in langzame, tonische of niet-twitch vezels. Deze vezeltypen vertonen zowel morfologische als fysiologische verschillen. Een iets ouder overzichtsartikel (Hess, 1970) legt de nadruk op de morfologische verschillen, terwijl een zeer recent overzichtsartikel (Morgan and Proske, 1984) de nadruk legt op de fysiologische verschillen waaraan de beide groepen hun naam ontlenen. Door Vrbova et al.(1978) wordt vooral ingegaan op de verschillen in innervatie tussen beide typen en op de gevolgen die dit voor de eigenschappen van de spiervezelmembraan heeft.

Tonische spiervezels reageren slechts op herhaalde stimuli; ze vertonen dan een langzame contractie. Verder reageren deze spiervezels op langdurige depolarisatie met een lang aangehouden, tonische, contractuur.
Fasische of twitch vezels reageren op een enkele stimulus van voldoende sterkte met een korte contractie, een twitch. Bij één langdurig aangehouden depolarisatie neemt de krachtsontwikkeling van deze vezels na enige tijd af.
De morfologische kenmerken van tonische spiervezels, zoals samengevat door Hess

(1970) zijn:
- Meerdere eindplaten per spiervezel.
- Weinig sarcoplasmatisch reticulum. De myofibrillen zijn hierdoor niet volledig van elkaar gescheiden (Felderstruktur, in tegenstelling tot de Fibrillenstruktur van twitch vezels; Kruger, 1929, geciteerd door Hess, 1970)
- Weinig contact tussen T systeem en sarcoplasmatisch reticulum; het contact-oppervlak is in tonische spiervezels 5 à 10 maal kleiner dan in twitch spiervezels (Page, 1965; Flitney, 1971).
- Afwezigheid van de M lijn.
- Dikke golvende Z lijnen.

Fasische vezels reageren op een depolarisatie die een bepaalde drempelwaarde overschrijdt met het generen van een actiepotentiaal. Tonische vezels generen, onder fysiologische omstandigheden, geen actiepotentiaal; een depolarisatie heeft alleen plaatselijk effect over een afstand en gedurende een tijd die afhangen van de kabeleigenschappen van de membraan: de capaciteit van de membraan en de weerstanden binnen, buiten en over de membraan.
Tonische spieren hebben een lage myosine ATPase activiteit (Bárány, 1967). Hierdoor is de krachtsontwikkeling langzaam, maar deze spieren verbruiken per tijdseenheid relatief weinig ATP (Goldspink, 1977). Deze spieren functioneren niet zo zeer bij bewegingen als wel bij het in standhouden van een positie. Het zijn houdings-spieren. De tonische ALD (m. anterior latissimus dorsi) van vogels houdt in rust de vleugels tegen het lichaam.

Tonische spiervezels komen bij alle klassen van gewervelde dieren voor. De bovengenoemde combinatie van eigenschappen is opgesteld aan de hand van onderzoek aan spiervezels van amfibiën. Bij "tonische" spiervezels van reptielen, vogels en vissen kunnen afwijkende combinaties van eigenschappen voorkomen.
Bij zoogdieren komen tonische spiervezels (uitgezonderd die in spierspoeltjes) alleen voor in oogspieren en in de m. tensor tympani. De overgrote meerderheid van de dwarsgestreepte spiervezels van zoogdieren zijn twitch vezels. Dat betekent geenszins dat het uitsluitend snelle vezels zijn.

3.2. Onderverdeling van fasische of twitch vezels

Bij zoogdieren worden drie verschillende typen twitch vezels onderscheiden: snelle witte, snelle rode en langzame rode. Hiervoor zijn verschillende combinaties van namen in gebruik (zie Goldspink, 1977, p 15). Daar in de meeste spieren deze typen gemengd voorkomen, zijn histochemische methoden om ze te onderscheiden onontbeerlijk.

Snelle en langzame vezels worden onderscheiden met behulp van immuun-histochemische reacties tegen de betreffende spiereiwitten en met histochemische reacties op de myosine-ATPase activiteit (Pool, 1980; te Kronnie, 1982). Daar de pH stabiliteit van het myosine ATPase een scherpere onderscheiding geeft, wordt deze meestal als maat gebruikt. Het myosine ATPase van langzame spiervezels is niet bestand tegen hoge pH (10.4), dat van snelle spiervezels is niet bestand tegen lage pH (4.35, Guth and Samaha, 1969).

De kleurtegenstelling rood-wit is gerelateerd aan het uithoudingsvermogen van de spier. Rode spiervezels danken hun kleur aan de aanwezigheid van myoglobine, een eiwit dat zuurstof bindt. De energie die deze vezels tijdens spieractiviteit verbruiken, wordt voor een groot deel geleverd door het aërobe metabolisme. De energie die witte vezels tijdens spieractiviteit verbruiken is voornamelijk afkomstig van de glycolyse; deze spiervezels hebben een minder goed uithoudingsvermogen dan rode spiervezels. Het enzym-histochemische onderscheid tussen rode en witte spiervezels berust dan ook op de activiteit van tot het metabolisme behorende enzymen. Daar rode spiervezels meer mitochondria hebben dan witte vezels, is het onderscheid tussen deze typen

ook in morfologisch opzicht goed mogelijk.

De histochemische reactie op myosine ATPase is, als criterium voor het onderscheiden van vezeltypen, pas tegen 1970 algemeen in gebruik geraakt. Voor die tijd is gepoogd snelle en langzame spiervezels te onderscheiden op grond van verschillen in het aantal en de verdeling van de mitochondria en de daarbij behorende enzymactiviteit. Dit heeft geleid tot een groot aantal indelingen die niet met elkaar in overeenstemming zijn (zie voor een overzicht Close, 1972). De nasleep van deze verwarring is nog te vinden in tamelijk recente tabellen met eigenschappen van de drie vezeltypen (Goldspink 1977, p. 15).

3.3. Spiervezels van vissen: fasisch of tonisch?

Spiervezels van vissen passen niet in de indeling fasisch-tonisch. Bij vissen wordt algemeen een indeling rood-wit gebruikt.

Witte spiervezels van vissen zijn op grond van hun innervatie onder te verdelen in twee categorieën. De eerste bestaat uit spiervezels die alleen aan de uiteinden geïnnerveerd zijn en een actiepotentiaal voortgeleiden (Barets, 1961; Bone, 1964).Teleostei waarbij deze wijze van innervatie voorkomt, worden door Bone (1978) als primitief beschouwd. De andere categorie bestaat uit spiervezels waarbij een aantal zenuweindigingen verdeeld over de hele lengte van de vezel voorkomt (multiterminale innervatie, Bone, 1978; Altringham and Johnston, 1978; zie ook hoofdstuk III). Waarschijnlijk wordt zo'n spiervezel zelfs door meerdere axonen geïnnerveerd (polyneurale innervatie) (Hudson, 1969). Spiervezels van de baars en de karper behoren tot deze tweede categorie. Deze spiervezels kunnen een actiepotentiaal genereren en reageren met een twitch op een enkele stimulus (Barets, 1961; Takeuchi, 1959; Hudson, 1969; Hidaka en Toida, 1969; zie ook de discussie van hoofdstuk IV). Rode spiervezels zijn altijd meervoudig geïnnerveerd; ze genereren geen actiepotentiaal. Dit type reageert op langdurige depolarisatie met een aangehouden contractie, een kenmerk van tonische vezels (Barets, 1961; Hidaka en Toida, 1969). Barets (1961) en Flitney en Johnston (1979) beschreven rode spiervezels van vissen (teleosten), die niet op een enkele stimulus reageren met een twitch.
De ultrastructuur van rode spiervezels van vissen komt echter meer overeen met die van twitch vezels; ze bezitten duidelijke M lijnen, duidelijk afgegrensde myofibrillen en een uitgebreid sarcoplasmatisch reticulum (Nishihara, 1967; Nakajima, 1969). Johnston (1981) vond dat rode spiervezels van de haai *Scyliorhinus canicula* op een enkele stimulus reageren met een twitch. Dat ook rode spiervezels van teleosten met een twitch reageren op een enkele stimulus bleek voor het eerst uit in dit proefschrift beschreven onderzoek (hoofdstuk IV).

Door hun functie bij langzame, langdurige bewegingen verschillen de rode spiervezels van vissen ook van de tonische spiervezels van amfibiën en vogels, die typische houdings-spieren zijn.

4. SPIERVEZELTYPEN BIJ VISSEN; relatie tussen structurele en contractiele eigenschappen

4.1. De vissen

Het onderzoek is verricht aan spieren van de baars en van de karper. In eerste instantie is voor de baars gekozen, omdat bij deze vis reeds een uitgebreide kennis van de relatie tussen positie, grootte, origo en insertie en

functie van de kopmusculatuur bestond (Osse, 1969). Onderzoek naar het voorkomen en de functie van spiervezeltypen sloot hier goed bij aan. De karper is gekozen omdat dit een in de visteelt, vooral in Oost Europa, belangrijke soort is. Deze vis, die inmiddels, vooral ten behoeve van immunologisch onderzoek, in ons laboratorium wordt gekweekt, is zeer geschikt om mee te experimenteren. Onderzoek aan de karper sluit bovendien goed aan op binnen de vakgroep verricht functioneel morfologisch onderzoek (Sibbing, 1982) en op elders (Groningen en Luik) verricht fysiologisch en biochemisch onderzoek (Ballintijn, et al., 1972; VandeWalle et al., 1983; Hamoir et al., 1981; Focant et al., 1981).

Het werken aan spieren van twee vissen met geheel verschillende levensgewoonten (carnivoor en omnivoor) biedt bovendien de mogelijkheid tot vergelijking van de vezeltype-eigenschappen van beide vissen.

4.2. Karakterisering van spiervezeltypen

De afgelopen jaren zijn er bij vissen meerdere typen spiervezels onderscheiden (Patterson et al.,1975; Mosse and Hudson, 1977; Korneliussen et al., 1978; Akster en Osse, 1978; van Raamsdonk et al.,1980; van Raamsdonk et al., 1982). Deze typen zijn alle onder te brengen in twee hoofdgroepen: rood en wit, die onderscheiden worden op grond van verschillen in de contractiele eiwitten (Focant et al.,1976; Pool et al.,1976). Op grond van verschillen in de pH stabiliteit van het myosine ATPase zijn zowel rode als witte subtypen te onderscheiden.
Een probleem dat zich hierbij voordoet,is het feit dat bij spiervezels van vissen de pH stabiliteit van het myosine ATPase een minder betrouwbaar criterium is dan bij zoogdieren (zie de discussie van hoofdstuk III). Dankzij de door van Raamsdonk et al. ontwikkelde, en welwillend ter beschikking gestelde, antisera tegen verschillende typen spiereiwitten was het mogelijk een betrouwbaar onderscheid te maken tussen de rode en de witte hoofdgroep. Verder werd ter herkenning van vezeltypen gebruik gemaakt van de bekende onderlinge positie van rode, rose (een wit sub-type) en witte vezels in de laterale rompspieren. Hierdoor konden,per vis, de verschillen in pH stabiliteit van het myosine ATPase tussen deze typen worden onderscheiden en ook op de kopspieren worden toegepast.

In de rompspieren gaan verschillen in de eigenschappen van de contractiele eiwitten en het myosine ATPase meestal samen met verschillen in het metabolisme en in de myoglobineconcentratie waaraan de vezeltypen rood, rose en

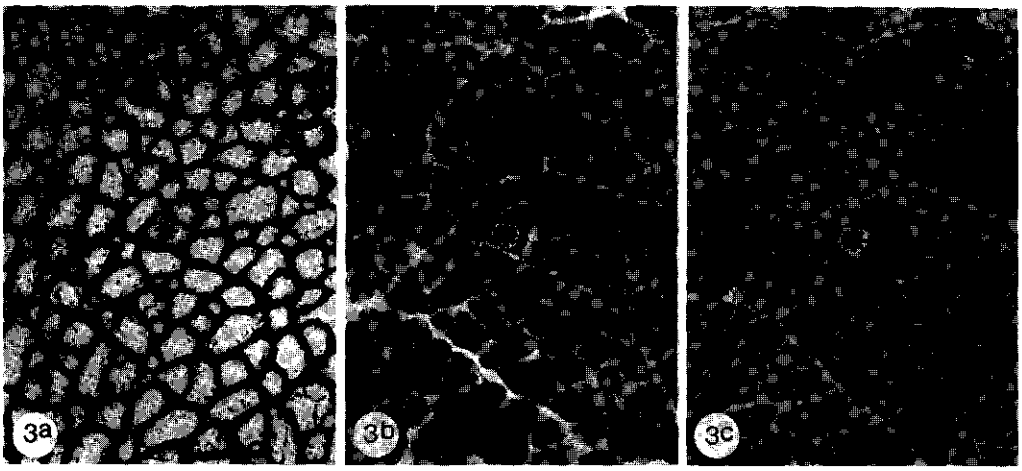

Fig. 3. Opeenvolgende dwarsdoorsneden door spiervezels uit de m. levator arcus branchialis V van de karper.
a) succinaatdehydrogenase activiteit
b,c) pH stabiliteit van het myosine ATPase
b) zure preincubatie,pH 4.4: rode vezels actief (donker); rose vezels gedeactiveerd (licht).
c) alkalische preincubatie, pH10.3; rose vezels actief, rode vezels gedeactiveerd.
Het sterretje staat in een rose vezel.

wit oorspronkelijk hun naam ontlenen.
In de kopspieren is dit veel minder het geval (Hamoir et al.,1981). Kopspiervezels die op grond van de pH stabiliteit van hun myosine ATPase als rood en rose worden gekarakteriseerd, zijn soms wat betreft hun succinaatdehydrogenase activiteit, niet van elkaar te onderscheiden (zie fig. 3).

Op grond van verschillen in de pH stabiliteit van het myosine ATPase werden zowel bij de baars als bij de karper vier typen spiervezels onderscheiden (hoofdstuk I en hoofdstuk II). Bij de baars kunnen deze typen op grond van verschillen in het metabolisme verder worden onderverdeeld. De vier onderscheiden typen werden bij beide vissen in meerdere spieren onderscheiden (hoofdstuk I; Akster en Sibbing, 1982). Daar verschillen in de pH stabiliteit van het myosine ATPase slechts indirect in verband kunnen worden gebracht met verschillen in contractiesnelheid is nagegaan in hoeverre histochemische verschillen tussen spiervezeltypen overeenkwamen met ultrastructurele kenmerken (hoofdstuk II en V). Ook werd nagegaan of er overeenkomst in ultrastructuur bestond tussen vezels van eenzelfde type, afkomstig uit verschillende spieren

(hoofdstuk II). In beide gevallen bleek er een duidelijke overeenkomst te bestaan.

Het meeste onderzoek aan spieren en spiervezeltypen van vissen betreft de axiale musculatuur. Hierover zijn overzichtsartikelen geschreven door Bone (1978) en Johnston (1981).

Van de auteurs die over kopspieren hebben gepubliceerd, karakteriseren Hughes en Johnston (1979) bij *Salmo gairdneri* de vezeltypen eveneens op grond van, in de rompspieren vastgestelde, verschillen in de pH stabiliteit van het myosine ATPase. Barends (1979) legt vooral de nadruk op verschillen in het metabolisme van spierdelen met een verschillende functie en op de embryonale ontwikkeling (Barends et al., 1983). Ohja en Datta Munshi (1975) beperken zich tot de verschillen in succinaatdehydrogenase activiteit. Dana Ono en Kaufman (1983) bepalen voor de pharyngeale spieren van een aantal vissen de pH stabiliteit van het myosine ATPase, delen dan echter in op macroscopische kleurverschillen, en komen zo tot een afwijkende vezeltype benaming.

4.3. Meting van de ultrastructuur

In de eerste, kwalitatieve studies over de ultrastructuur van spiervezels van vissen werden geen verschillen gevonden, die gerelateerd konden worden aan verschillen in contractiesnelheid (Nishihara, 1967; Nakajima, 1969). Kwantitatieve metingen tonen aan dat die verschillen er wel degelijk zijn (zie tabel 1 en hoofdstuk VI).

Een veel gebruikte, snelle en nauwkeurige methode om uit twee-dimensionale coupes kwantitatieve informatie over drie-dimensionale structuren te verkrijgen is de stereologie. Hierbij wordt gebruik gemaakt van de kwantitatieve relatie die bestaat tussen de gemiddelde afmeting van een groot aantal organellen en de gemiddelde afmeting van de doorsneden van deze organellen in coupes (Weibel en Bolender, 1973). De doorsneden die in een bepaalde coupe zijn getroffen, kunnen worden beschouwd als een steekproef. De afmetingen hiervan worden bepaald met behulp van een rooster dat op een (electronenmicroscopische) opname van de coupe wordt gelegd. Dit is te beschouwen als een nieuwe steekproef. Het relatieve volume van een bepaald organel is na te gaan aan het percentage roosterpunten dat op de doorsneden van dat organel valt. Het relatieve oppervlak is te berekenen uit het aantal malen dat de roosterlijn de omtrek van de organel-doorsneden snijdt (zie voor de hierbij gebruikte formules materiaal en methoden van hoofdstuk VI). Stereologische studies van spiervezels worden gecompliceerd door de hoge mate van oriëntatie

Tabel I. Relatieve volumes en oppervlakken van SR en T systeem bij enige vissen, amfibien en zoogdieren.

		SR						T systeem					
	auteur	volume %			oppervlak μ^{-1}			volume %			oppervlak μ^{-1}		
Vis		rood	rose	wit	rood	rose	wit	rood	rose	wit	rood	rose	wit
Salmo gairdneri	Nag, 1972	5,1	-	13,7	1,44	-	2,42	0,1	-	0,4	0,16	-	0,36
Gadus virens	Patterson en Goldspink, 1972	4,6*		7,2*									
Etmopterus spinax	Kryvi, 1977	4,9	8,0	6,0				0,33	0,56	0,50			
Galeus melastomus	Kryvi, 1977	4,6	5,0	6,8				0,57	0,68	0,89			
Carassius auratus	Penney en Goldspink, 1980			9-14[a]			0,9-2,5[a]						
Mugil cephalus	Quaglia, 1980	7,2*		8,3*									
Anquilla anquilla	Egginton en Johnston, 1982	2,1	6,8	4,7-5,6[b]				0,31	0,37	0,30-0,44[b]			
Engraulis encrasicolus	Johnston, 1982	2,7						0,43					
Cyprinus carpio	Akster, Hdst. V	5,4*	4,4*		3,2*	2,8*							
Perca fluviatilis	Akster et al.[c]	3,7	4,6	5,3	2,7	2,6	2,7	0,17	0,30	0,26	0,07	0,19	0,15
Amfibien													
Ambistoma mexicana	Totland 1976	2,5*	4,5*	11,7*									
Rana temporaria	Flitney, 1971							tonisch 0,18		fasisch 0,39			
Rana pipiens	Mobley en Eisenberg, 1975			fasisch 10,9			fasisch 2,43			0,36			fasisch 0,27
Zoogdieren		SO	FOG	FG	SO	FOG	FG	SO	FOG	FG	SO	FOG	FG
Cavia	Eisenberg en Kuda 1976	3,6	4,0	5,6	1,09	1,22	1,62	0,16	0,35	0,33	0,07	0,18	0,18
Kat	van Winkle et al. 1978	3*	5,5*	6,5*									

* SR en T systeem gecombineerd bepaald

a) afhankelijk van temperatuuracclimatisatie

b) meerdere typen

c) (Hdst. II, VI en unpubl. results)

SO, slow oxidative

FOG, fast oxidative glycolytic

FG, fast glycolytic

van dit weefsel. In het in dit proefschrift beschreven onderzoek werd gebruik gemaakt van de hiervoor door Eisenberg et al. (1974) ontwikkelde correctiefactoren.

4.4. Meting van contractiele eigenschappen

Het meten van de contractiele eigenschappen van multiterminaal geïnnerveerde spiervezels, waarvan in ieder geval de rode vezels geen actiepotentiaal voortgeleiden, werd bij de karperspieren gedaan aan de hand van een spierzenuw preparaat. De spiervezels van de baars werden direct gestimuleerd met parallel aan de vezelbundels geplaatste electroden. Met deze beide methoden werden vergelijkbare resultaten verkregen.
Het uithoudingsvermogen van rode en rose spiervezels van de karper werd bepaald door deze spiervezels,met een vaste frequentie, herhaaldelijk in tetanus te brengen (volgens Burke et al.,1973).

4.5. De relatie tussen ultrastructurele en contractiele eigenschappen

De verschillen in het uithoudingsvermogen van rode en rose spiervezels van de karper komen overeen met verschillen in het relatieve volume van de mitochondria en in de, kwalitatief waargenomen, hoeveelheid glycogeen.

Het ultrastructurele kenmerk dat de beste relatie vertoont met de verschillen in stijgtijd van de isometrische twitch is de relatieve lengte aan contact tussen het T systeem en het SR (zie hoofdstuk VI). Het kenmerk dat meestal bepaald wordt,is het relatieve volume van het sarcoplasmatisch reticulum (zie tabel 1). Somlyo et al.(1979) tonen echter aan dat juist het relatieve volume, vooral dat van de longitudinale tubuli, sterk wordt beinvloed door fixatie artefacten. Uit de hoofdstukken IV, V en VI blijkt dat ook het relatieve oppervlak van het sarcoplasmatisch reticulum niet gerelateerd hoeft te zijn aan de contractiesnelheid.

Een kenmerk waarvan ook verondersteld werd dat het gerelateerd was aan snelheidsverschillen tussen typen spiervezels (Davey et al.,1975) is de ligging van het T systeem: bij de Z lijn (een keer per sarcomeer) of aan de uiteinden van de A band (twee keer per sarcomeer). Uit hoofdstuk II blijkt dat in de onderzochte spiervezels de ligging van het T systeem geen verband houdt met de uitgebreidheid van het contact tussen T systeem en SR, maar met de lengte van de actine filamenten. Het verschil in lengte-spanningscurve tussen sarcomeren met korte en met lange actinefilamenten, dat in hoofdstuk II op grond van de sliding filament-cross bridge theorie werd voorspeld, blijkt ook

experimenteel te worden gevonden (hoofdstuk VII).

4.6. Het functioneren van spiervezeltypen in de vis

Uit het bovenstaande blijkt dat een spier als de m. levator operculi van de baars vier typen spiervezels bevat, die verschillen in contractiesnelheid, uithoudingsvermogen en in lengte-spanningscurve en derhalve specifiek geschikt zijn voor verschillende typen van beweging. Uit electromyogrammen blijkt dat het laterale deel van deze spier, waarin uitsluitend één van deze typen voorkomt, actief is tijdens zeer langzame beweging, terwijl het deel van de spier dat de drie overige typen bevat, actief is bij meer incidentele, snelle bewegingen.

Hoe de vissen de contractiesterkte regelen van hun multiterminaal en polyneuraal geïnnerveerde spiervezels is een belangrijke, maar vooralsnog onbeantwoorde vraag.

Een met de levenswijze samenhangende verdeling van de diverse typen spiervezels, zoals door Boddeke, Slijper en van der Stelt (1959) is beschreven voor de zwemspieren van baars en karper, wordt ook gevonden in de kopspieren. De kopspieren van de baars bevatten relatief meer witte spiervezels dan die van de karper (hoofdstuk I, Barends, 1979; Akster en Sibbing, 1982). Witte spiervezels van de rompspieren van de baars hebben een meer uitgebreid contact tussen het T systeem en het sarcoplasmatisch reticulum dan die van de karper. Op grond hiervan is te verwachten dat de witte axiale musculatuur van de baars sneller is dan die van de karper. Beide verschillen komen overeen met het verschil in levenswijze tussen de baars, een predator die prooi achtervolgt en met snelle beweging opzuigt en de karper, een omnivore vis met een rustiger wijze van voedselopname.

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MUSCLE FIBRE TYPES IN HEAD MUSCLES OF THE PERCH PERCA FLUVIATILIS (L), TELEOSTEI

A HISTOCHEMICAL AND ELECTROMYOGRAPHICAL STUDY

by

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SUMMARY

In head and body muscles of the perch four main fibre types, two white and two red, can be distinguished using ATPase activity, pH stability of ATPase and reactions with specific antisera. On the basis of SDH activity and LDH activity each red type was subdivided in two subtypes. One of these subtypes is possibly a differentiating fibre. Electromyographic recordings from head muscle parts containing white fibre types only show activity during rapid vigorous movements (eating, coughing). Muscle parts containing red types are also active during respiratory movements.

Fibres corresponding to the well-known description of red muscle fibres of fish are only found in the body muscles. The red head muscles differ from this type by a high LDH activity or by a high, alkali stable, acid stable ATPase activity as determined by histochemistry.

A red muscle part consisting mainly of these "high ATPase" red fibres showed only EMG activity during increased respiration. During quiet respiration EMG activity was recorded from a muscle part with a high proportion of red fibres with low ATPase activity and high LDH activity. It is not only in body muscles but also in head muscles that the distribution and occurrence of muscle fibre types is closely related to the functional demands imposed on the muscles during the life of the fish.

Key words: muscle fibre types, ATPase, electromyography, immuno histochemistry, fish.

INTRODUCTION

Classification of muscle fibres is generally based on criteria correlated with their speed of contraction and on criteria correlated with their resistance to fatigue.

Speed of contraction is correlated with the level and the pH stability of myofibrillar ATPase activity. The occurrence of the enzymes of aerobic metabolism is correlated with resistance to fatigue (BARANY, 1967; PETER *et al.*, 1972; BURKE *et al.*, 1973).

In the segmental body muscles of fishes different fibre types have been described. The bulk of these muscles consists of white fibres showing the features belonging to an anaerobic metabolism and a high,

alkali stable ATPase activity; under the lateral line a V-shaped narrow superficial strip of red muscle is present. These fibres have the characteristics of an aerobic metabolism and a low, alkali labile ATPase activity (NAG, 1972; JOHNSTON *et al.*, 1972; POOL *et al.*, 1976). In some cases pink fibres with properties intermediate between those of the red and the white fibres were found wedged between the red and white zones (JOHNSTON *et al.*, 1974; JOHNSTON *et al.*, 1975; PATTERSON *et al.*, 1975; JOHNSTON *et al.*, 1977). At low swimming speeds only red fibres are used. White fibres are active during bursts of activity and at high cruising speeds (BONE, 1966; HUDSON, 1973; JOHNSTON, et al 1977).

The present study was undertaken to test the hypothesis that the functional demands of fish head muscles are reflected in their fibre composition. Muscles used for the fast, short duration movements of prey catching were expected to show an anaerobic metabolism and a high, alkali stable, acid labile ATPase. In muscles active during the slow, sustained respiratory movements an aerobic metabolism and a low, acid stable, alkali labile ATPase activity were assumed. For this test the musc. levator operculi anterior and the musc. levator operculi posterior (Fig. 1), small muscles that participate in opening the mouth by lowering the lower jaw during the respiratory movement, were selected because of their clear role, their small dimensions and their accessibility for EMG measurements. These muscles also show EMG activity during jaw movements while feeding, but in this movement the

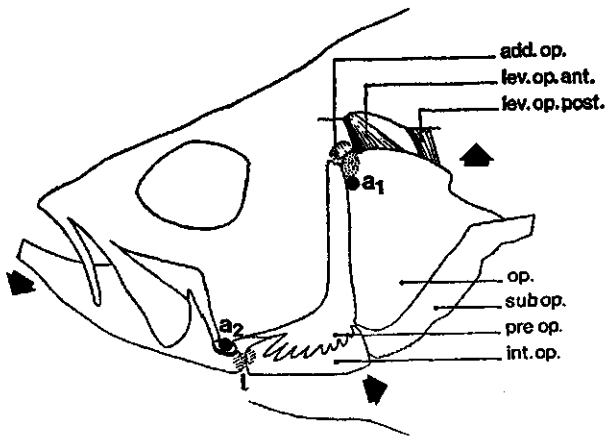


Fig. 1. Localization of the adductor operculi, the levator operculi anterior and the levator operculi posterior. Rotation of the opercular complex, brought about by contraction of both levators, is transmitted to the lower jaw by the interopercular-mandibular ligament and results in the lowering of the lower jaw. (see arrows). a_1: hyomandibular-opercular articulation; a_2: articulation of lower jaw; op: operculum; subop.: suboperculum; preop.: preoperculum; int. op.: interoperculum.

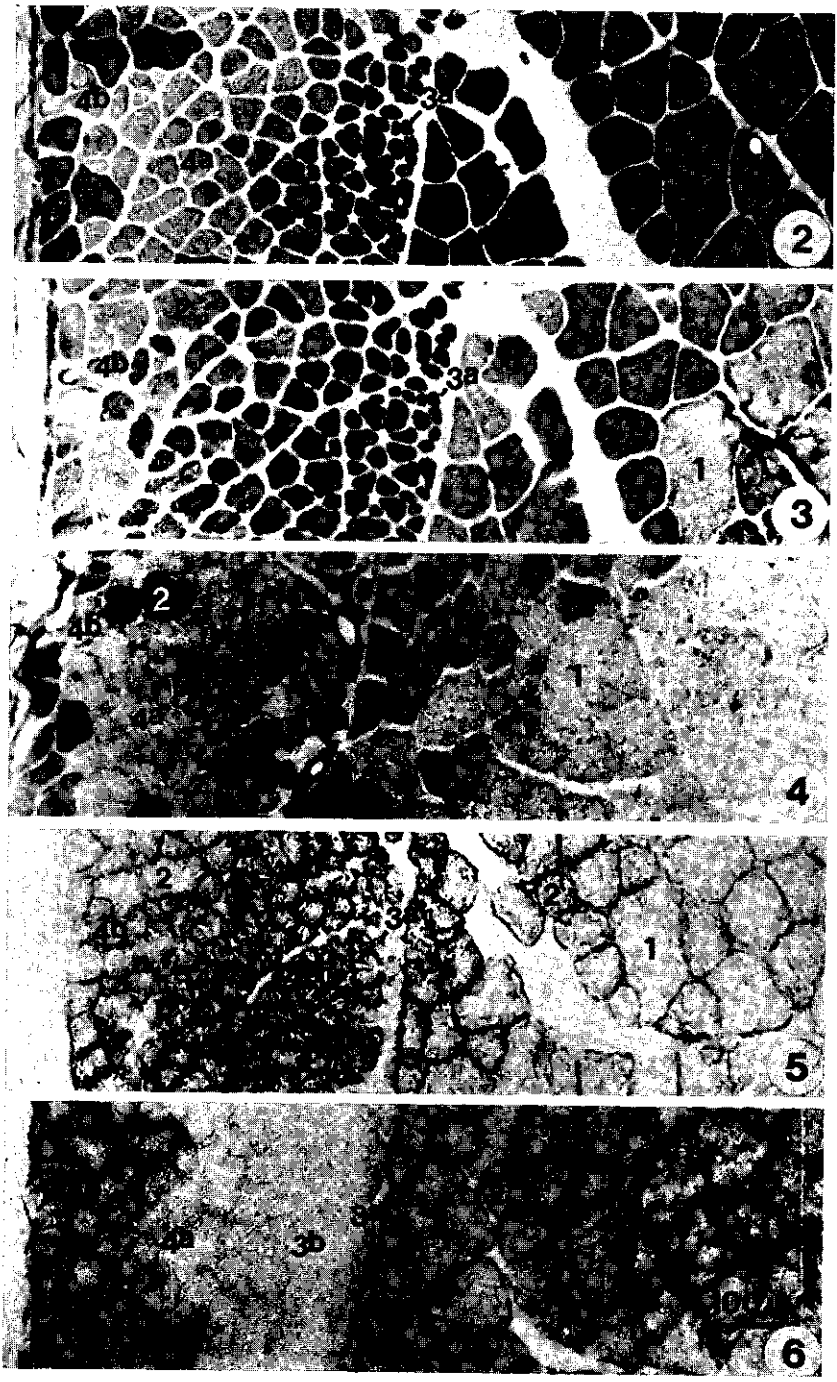
main work is done by the sternohyoideus (Osse, 1969). A histochemical investigation of the sternohyoideus is therefore included. At a later stage of the study the fibre composition of the red part of the adductor operculi was also investigated. Since the body muscles of fishes have already been well studied a strip of body muscle was also investigated to serve as a permanent reference.

MATERIAL AND METHODS

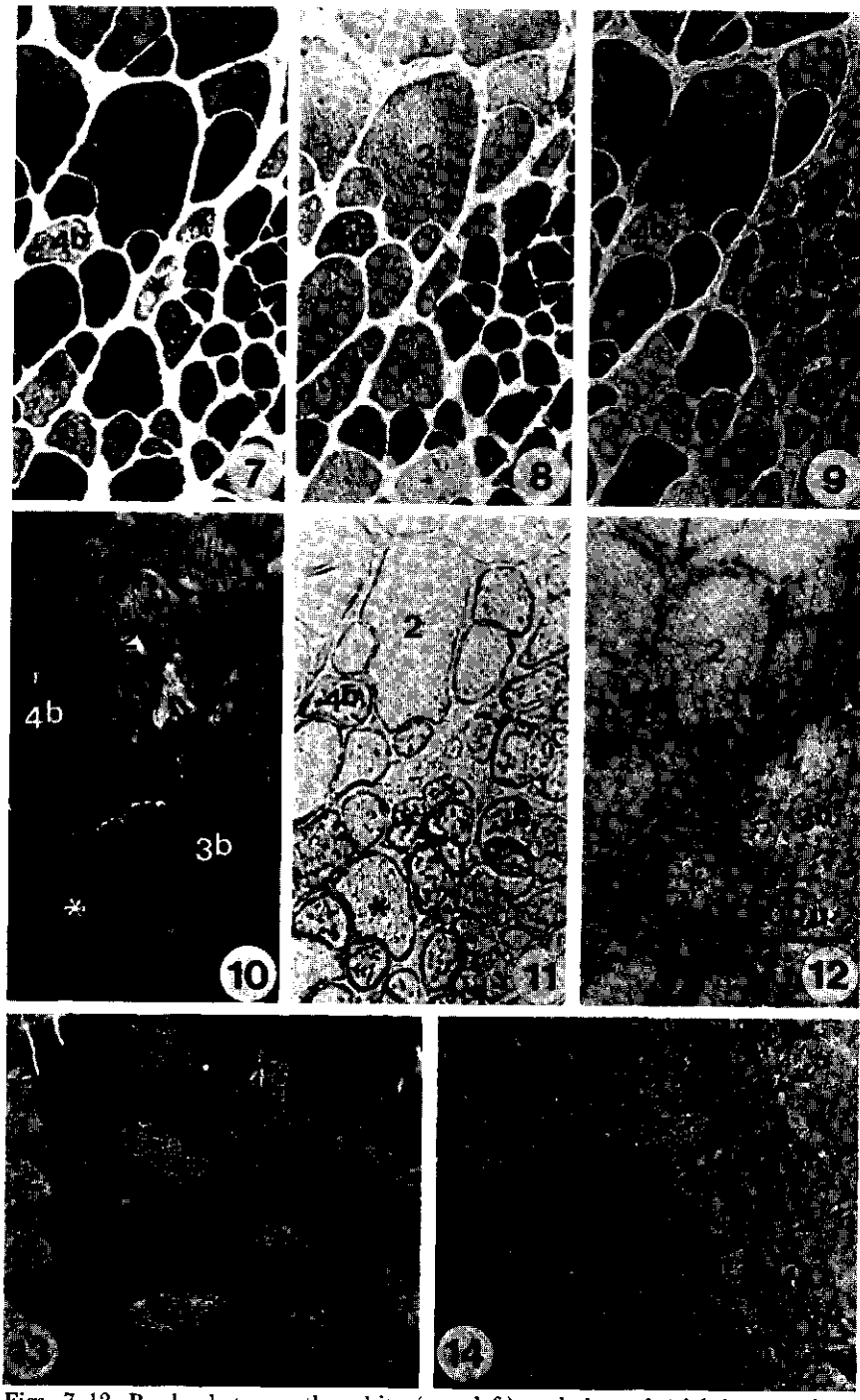
Perch, *Perca fluviatilis* (L), measuring 19–25 cm, caught in the lake district in the west of the Netherlands were obtained from commercial fishermen and kept in tanks until needed.

Histochemistry. Muscles dissected from fish anaesthetized with 0.2% MS 222, were frozen in liquid freon or isopropane. The tissue was processed immediately after dissection or stored at —90° C until required. Each levator was sectioned and processed together with a strip of lateral body muscle, comprising both red and white fibres from the level of the second dorsal fin. The sternohyoideus and the adductor operculi were sectioned separately. The test with the antisera was not done on the adductor operculi. Each muscle was examined in at least three fishes. Unfixed tissue was used for all histochemical tests. For the demonstration of ATPase activity a modified method of the one described by Guth & Samaha (1969, 1970) was used. Incubations were carried out at a room temperature of 20–22° C. Incubation time in the substrate was 20 min. Acid pre-incubation time was 2 min at pH 4.5 and alkaline pre-incubation time was 2 min at pH 10.0 or pH 10.2. Succinate dehydrogenase (SDH) activity was determined using the method described by Nachlas *et al.* (1957). The incubation medium for lactate dehydrogenase (LDH) consisted of: 0.5 ml 0.1M Lithium lactate, 2.8 ml Trisbuffer pH 7.3, 3 ml aqua dest., 0.3 ml 0.075% Phenazine methosulphate, 0.25 ml 0.1 M NaCN, 5 mg NAD, 4 mg Nitro Blue Tetrazolium. To prevent diffusion of this soluble enzyme 100 mg/ml polyvinylalcohol was added to the substrate which was then applied on a celloidine membrane covering the sections. Incubation time was 15 min at 37° C. Immuno histochemistry was carried out using the method described by Pool *et al.* (1976) and with their antisera.

Electromyography was done on free swimming perch as described by Osse (1969). After the removal of a small piece of skin dorsal to the operculum it was possible to discern a white and a red part of the levator operculi anterior. Copper wire electrodes with a diameter of 50 μm were inserted into the red and white parts of the muscle and into the rostral part of the levator posterior while the fish was anaesthetized with



Figs. 2–6. Body muscle; area (corresponding to rectangle in Fig. 17) comprising red (left), pink and white (right) zones. Fibre types are numbered as in table 1. Fig. 2. ATPase activity after pre-incubation at pH 10.0. Fig. 3. ATPase activity after acid pre-incubation. Fig. 4. ATPase activity after alkaline pre-incubation at pH 10.2. Fig. 5. SDH activity. Fig. 6. LDH activity.



Figs. 7–12. Border between the white (top, left) and the red (right) part of the levator operculi anterior. Fibre types are numbered as in Table 1. On the border between the zones some aberrant fibres are present; asterisk: "pink fibre not reacting with antiserum against white myosin; pointed stars: red fibres that react with antiserum against white myosin. Fig. 7. ATPase activity. Fig. 8. ATPase activity after acid pre-incubation. Fig. 9. ATPase activity after pre-incubation at pH 10.2. Fig. 10. Reaction with antiserum against white myosin. Fig. 11. SDH activity. Fig. 12. LDH activity.

Figs. 13–14. Body muscles, reaction with antiserum against white actin. Fig. 13. Pink (light) fibres in the red (dark) zone. Fig. 14. Border between red and pink zone.

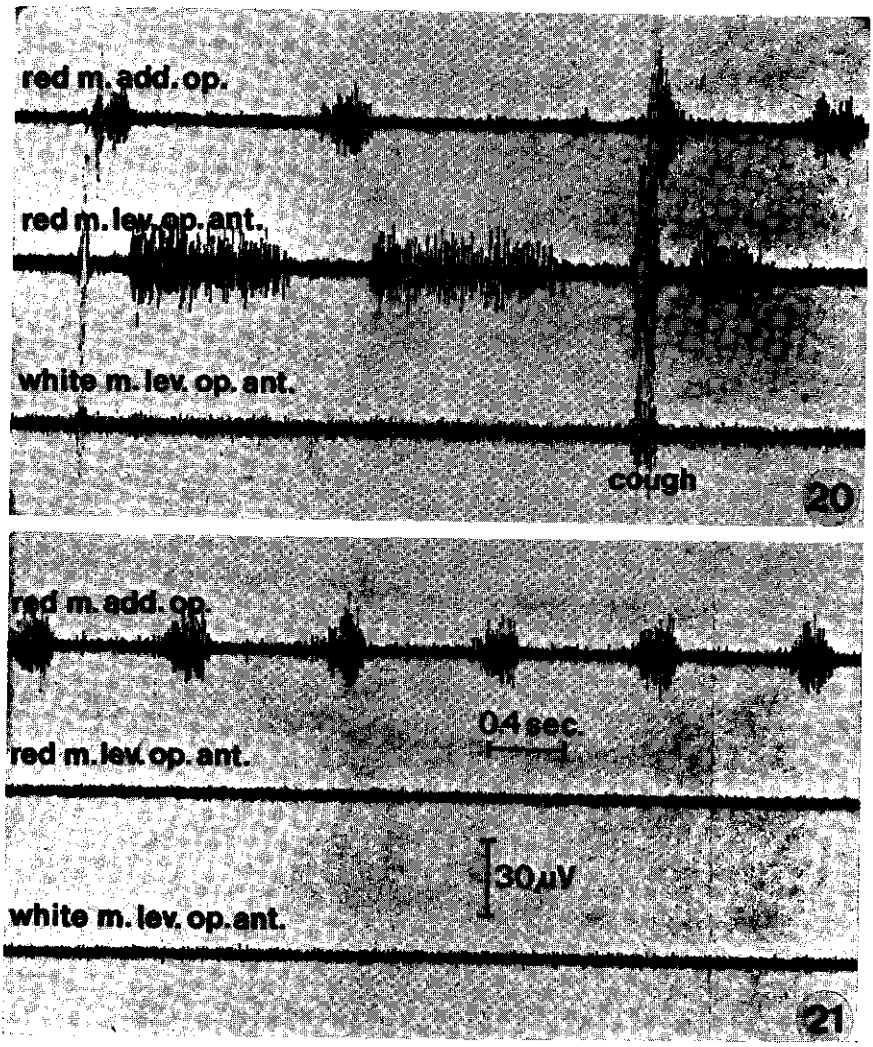
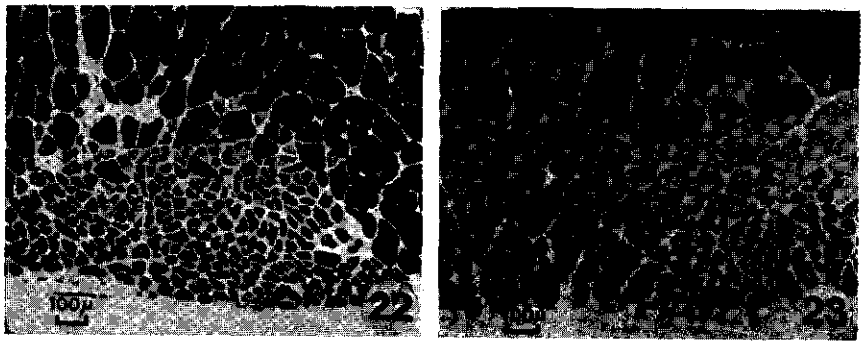


Fig. 20. Deep ventilation and coughing 2 hours after anaesthesia. The white muscle part is only active during coughing. The red muscle parts show rhythmic respiratory activity. For time and amplitude scales see Fig. 21.

Fig. 21. Quiet ventilation 6 hours after anaesthesia, when hardly any jaw movements are observed. The red part of the adductor operculi shows rhythmic activity. Activity in the levator operculi anterior has ceased. This is a typical pattern of EMG activity for a fish at rest.



Figs. 22 and 23. Red zones of the levator operculi anterior (Fig. 22) and the adductor operculi (Fig. 23), ATPase activity without pre-incubation. The red zone of the adductor operculi has a greater proportion of fibres with a low ATPase activity (4^b fibres) than the red zone of the levator operculi anterior.

0.1% MS 222. The position of the electrode tips could be inspected with a dissection microscope. Similar electrodes were inserted by means of a 0.3 mm diameter fork through the skin into a red part of the adductor operculi. This red muscle part could be seen through the thin translucent skin covering this muscle on the ventro-caudal side. For each muscle EMGs were obtained from at least 5 fishes.

RESULTS

In the body muscles of the perch, in both the levator operculi anterior and the adductor operculi a white and a red zone were macroscopically recognisable. The sternohyoideus was white. The levator operculi posterior also had a white appearance, but under a dissection microscope the presence of red strands was noted in the caudal and middle part of this muscle.

Histochemical reactions

Body muscles

ATPase. In the lateral body muscles incubation for ATPase without pre-incubation revealed a sharp border between the smaller mainly light-staining fibres of the red zone and the larger dark-staining pink and white fibres. On the medial side of the red zone a thin irregular strip of very small dark fibres occurred. In the most lateral part of the red zone some scattered larger dark fibres were seen. Pre-incubation at an alkaline pH up to pH 10.0 did not change this pattern (Fig. 2). After pre-incubation at pH 10.2 the white fibres were inactivated. The pink fibres, situated between the red and the white zones and the fibres lateral in the red zone still stained dark. The small fibres with high ATPase activity medial in the red zone remained rather dark after pre-incubation at pH 10.2 (Fig. 4). These latter fibres also stained dark after acid pre-incubation. This colour remained even when incubation for ATPase was preceded by both acid and alkaline pre-incubation. All other fibres stained pale after acid pre-incubation (Fig. 3).

Immuno fluorescence. The reactions with antisera against white muscle proteins also revealed a sharp distinction between the red zone and the pink and white zones (Fig. 14). The small fibres with acid stable, alkali stable ATPase at the medial side of the red zone showed no reaction with the antisera. The fibres with high ATPase lateral in the red zone did react with the antisera (Fig. 13).

SDH. The white fibres had a low SDH activity. Most fibres of the red zone had a high SDH activity. In the pink fibres a gradient was seen: fibres close to the red zone had a higher SDH activity than fibres

close to the white zone. The fibres with high ATPase activity lateral in the red zone had an intermediate SDH activity. The SDH activity of the small fibres with high, alkali stable, acid stable ATPase activity medial in the red zone varied from low to rather high (Fig. 5).

LDH. In the red zone the fibres with a high, alkali stable, acid stable ATPase and most fibres with a low ATPase activity also had a low LDH activity. The fibres of the pink and white zones and the fibres with a high ATPase activity lateral in the red zone had a high LDH activity. Some fibres with a low ATPase activity situated between these lateral fibres with a high ATPase activity also had a high LDH activity (Fig. 6).

Head muscles

The levator operculi anterior consists of a lateral red portion partly surrounded by a much larger white zone. The red fibres insert just ventral to the dorsal edge of the operculum. Most fibres of the white zone originate from a medial aponeurosis found throughout three quarters of the muscle's length. They insert at the dorsal side of the ridge which lies on the medial side of the operculum.

ATPase. The red zone of this muscle mainly consists of fibres with a high ATPase activity. This ATPase had a good acid stability and a rather good alkali stability (Figs. 8 and 9). Close to the white zone a number of fibres with low ATPase activity is present (Figs. 7 and 22). Some fibres with low ATPase activity are also found scattered through the white zone. The white zone consists mainly of fibres with a high, acid labile ATPase (Figs. 7 and 8). After pre-incubation at pH 10.2 this area shows a mosaic of dark staining and inactivated fibres.

Immunofluorescence. Only the fibres of the white zone with high ATPase activity reacted with the antisera. The fibres with low ATPase activity and the fibres of the red zone with high, alkali stable, acid stable ATPase activity showed no reaction. On the border between the red and the white part some fibres deviated from this pattern (Fig. 10).

SDH. Most fibres of the red zone with a high, alkali stable, acid stable ATPase had a high SDH activity, but a small number of these fibres with high ATPase activity had a low or intermediate SDH activity. Fibres with a low ATPase activity, both those in the red zone and those scattered through the white zone, had a high SDH activity. The other fibres of the white zone had a low or fairly low SDH activity (Fig. 11).

LDH. All fibres found in the white zone and the fibres with a low ATPase activity had a high LDH activity. The red fibres with high acid stable, alkali stable ATPase had a low activity (Fig. 12).

In other head muscles similar relationships between the activities

of the different enzymes were found. The composition of these other head muscles is described after the description of the fibre types.

Fibre types

Fibres with identical or nearly identical patterns of enzyme activity were grouped together in fibre types according to the criteria shown in Fig. 15. Table 1 gives a survey of the enzyme activities found in the different fibre types. In Figs. 16–19 the localisation of the different fibre types in the body muscles and in both levators is shown. The numbers in the description below refer to the fibre type numbers in Table 1.

White fibres (type 1) showed fluorescence after treatment with antisera against white muscle proteins (Fig. 14) and contained a high ATPase activity that was resistant to alkaline pH up to pH 10.0 (Fig. 2). It disappeared after pre-incubation at pH 10.2 (Fig. 4) and after acid pre-incubation (Fig. 3). SDH activity in these fibres was low (Fig.

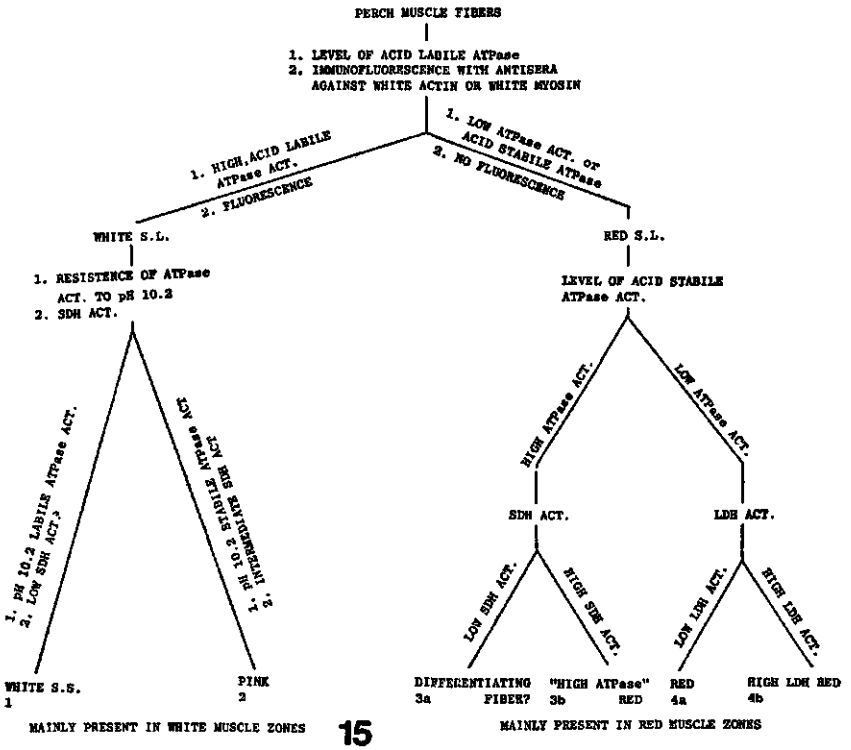


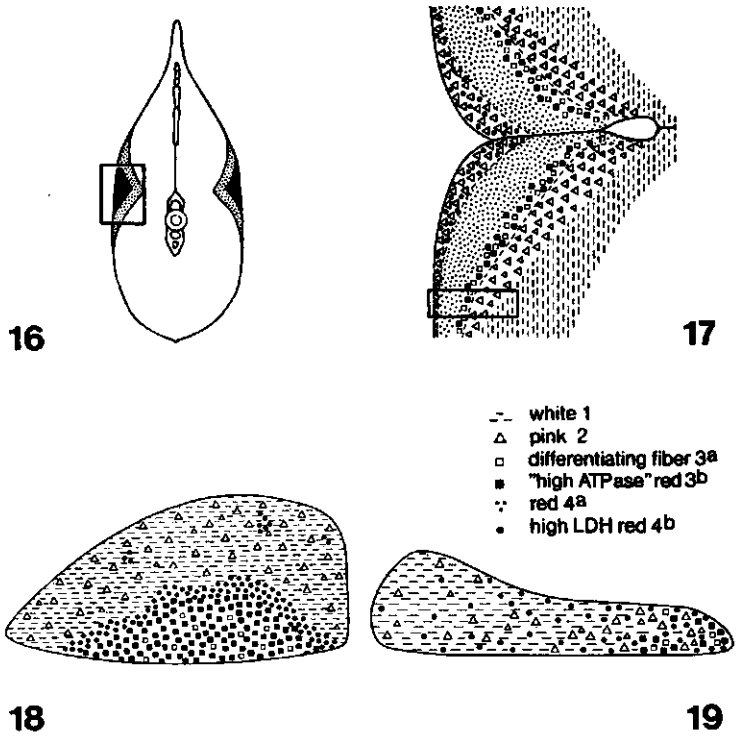
Fig. 15. Diagram of the fibre type classification.

TABLE I

Reaction with the antisera and enzyme activities of the different fibre types.

Fibre type	*Reaction with anti-white antisera*	*ATPase without pre-incubation*	*ATPase after pre-incubation at*			*SDH*	*LDH*
			pH 4.5	*pH 10.0*	*pH 10.2*		
1 white	+++	+++	−	+++	−	+	+++
2 pink	+++	+++	−	+++	+++	++	+++
3[a] differentiating fibre?	−	+++	+++	+++	++	+/++	+
3[b] "high ATPase" red	−	++/+++	++/+++	++/+++	+/++	+++	+
4[a] red	−	+	+	+	−	+++	+
4[b] high LDH red	−	+	+	+	−	+++	+++

−: no reaction or very low activity, +: low activity, ++: intermediate activity, +++: high activity.



Figs. 16–19. Diagrams of the localization of the muscle fibre types. Fig. 16. Perch tail, cross section, position of the red (dark) pink (dotted) and white muscle zones. Fig. 17. Area corresponding to rectangle in Fig. 16; rectangle in Fig. 17: area corresponding to Figs. 2–6. Fig. 18. Cross-section of the levator operculi anterior just dorsal to the rim of the operculum; left = rostral; top of the picture = medial. Fig. 19. Cross-section of the levator operculi posterior, left = rostral; top of the picture = medial.

5) and they had high LDH activity (Fig. 6). These fibres formed the bulk of the body muscles. Of the head muscles the sternohyoideus consisted mainly of these fibres and they formed the main component of the white zone of the adductor operculi. In the levator operculi anterior and posterior they were present in a mosaic pattern with the pink fibres (Figs. 18 and 19).

Pink fibres (type 2) also showed fluorescence after incubation with the antisera (Figs. 10 and 13). The high ATPase activity of these fibres survived pre-incubation at pH 10.2 (Figs. 4 and 9) and disappeared after acid pre-incubation (Figs. 3 and 8). These fibres had a high LDH activity (Fig. 6) and an intermediate SDH activity (Fig. 5). A gradient was seen in the SDH activity. Fibres close to the red zone

had a higher SDH activity than those close to the white zone. Pink fibres occurred in the zone between the red and the white parts of the body muscles (Fig. 17), laterally in the red zone of the body muscles and in the white zones of the head muscles (Figs. 18 and 19).

Red fibres of type 4^a correspond to the familiar descriptions of fish red muscle fibres. They showed no reaction with the antisera against white muscle proteins. (Figs. 13 and 14), had a high SDH activity (Fig. 5), a low LDH activity (Fig. 6) and a low, alkali labile ATPase activity (Figs. 2 and 4). After acid pre-incubation the ATPase activity of these fibres was unaffected (Fig. 3). They were present in the red zone of the body muscles but have not yet been found in the head muscles.

High LDH red fibres, type 4^b, differed from type 4^a by having a high LDH activity (Fig. 6). These fibres are present laterally in the red zone of the body muscles, intermingled with the scattered pink fibres (Fig. 17), and in the head muscles (Figs. 18 and 19).

Type 3^a fibres differed markedly from the type 4 fibres. They stained dark after incubation for ATPase. When incubation for ATPase was preceded by acid or by alkaline pre-incubation these fibres remained rather dark (Figs. 3 and 4) and if these procedures were both applied the result remained the same. They showed no fluorescence after incubation with the antisera.

SDH activity of these fibres was low or intermediate and LDH activity was low (Figs. 5 and 6) and they also had a very small diameter. The fibres were present as a series of small clusters deep in the red zone of the body muscles and, in low numbers, between the 3^b fibres in the red zones of the head muscles.

"High ATPase" red fibres, type 3^b did not generally show any fluorescence after incubation with the antisera (Fig. 10). Most 3^b fibres had a high ATPase activity (Fig. 7), but fibres with an activity intermediate between that of the type 3^a fibres and that of the type 4 fibres were also seen. This ATPase was acid stable and relatively alkali stable (Figs. 8 and 9). The fibres had a high SDH activity, (Fig. 11), and a low LDH activity (Fig. 12). They were found in the red zones of the head muscles and were also present in variable but low numbers on hte medial side of the red zone of the body muscles.

Aberrant fibres. In muscle parts with a distinct red or white character all fibres belong to one of the fibre types. But in the transition zones between the red and white zones of the head muscles some fibres occur, the characteristics of which are not wholly consistent with any one of the fibre types. This is mainly because of disagreements between the ATPase reactions and the reaction with the antisera (Figs. 7–10). Such deviations from the described pattern are also found among the pink fibres lateral in the red zone of the body muscles.

Composition of the other head muscles

The levator operculi posterior (Fig. 19) is a parallel fibred muscle consisting of several bundles of varying length. The anterior bundles inserting dorsally to the opercular ridge consist mainly of white (type 1) and pink (type 2) fibres. One or two red fibres with low ATPase activity are present (type 4^b). The fibre bundles gradually become shorter caudally and laterally while the proportion of red fibres, both those with low ATPase activity (type 4^b) and those with high, acid stable, alkali stable ATPase activity (type 3^b) increases. The most caudal bundle consists almost exclusively of red fibres with high, acid stable, alkali stable ATPase. Within the bundles the fibres usually run throughout the whole length of the bundle.

The sternohyoideus consists mainly of white (type 1) fibres. On the outside of the muscle some pink (type 2) fibres are found.

The adductor operculi. On the ventro-caudal side of this muscle a red zone occurs. This zone consists of a strip of red fibres with high ATPase activity (mainly type 3^b), on the outside of the muscle and a strip of high LDH red fibres (type 4^b) situated between the type 3 fibres and the white part of the muscle.

The latter consists mainly of white (type 1) fibres. Near the red zone some pink (type 2) fibres are present. The red part of the adductor operculi has a greater proportion of red fibres with low ATPase activity (type 4^b) than the red part of the levator operculi anterior (Figs. 22 and 23).

Electromyography

During rapid vigorous movements such as coughing, gaping and feeding and also during extreme hyperventilation, caused by perfusing the tank water with carbon dioxide, activity was recorded from white as well as from red muscle parts (cough in Fig. 20).

Red muscle parts were also active during respiration. The red part of the levator operculi anterior was active during the deep respiration when the fish was recovering from anaesthesia (Fig. 20), during respiration shortly after feeding and at moments when the fish seemed disturbed. During very quiet respiration, when the fish was fully recovered from anaesthesia and was at rest at the bottom of the tank, no activity was recorded from these fibres. In the red part of the adductor operculi activity was observed even during this quiet respiration (Figs. 20 and 21, Table 2).

TABLE II

EMG activity of red and white head muscle parts.

Muscle part	*Active during*			*Fibre types*
	fast movements (eating, coughing)	*deep respiration*	*very quiet respiration*	
sternohyoideus*	+	—	—	mainly white, some pink
white lev. op. post.	+	—	—	white and pink
white lev. op. ant.	+	—	—	white and pink
red lev. op. ant.	+	+	—	mainly "high ATPase" red
red add. operculi	+	+	+	"high ATPase" red and high LDH red

—: no EMG activity observed, +: EMG activity. *: EMG data of the sternohyoideus from Osse (1969).

DISCUSSION

In our study the main classification is based on the results of the ATP-ase reactions and the reactions with the antisera against white muscle proteins; reactions which are related to the contractile properties of the fibres (Fig. 15). These reactions yielded clear distinctions between positive and negative results and there was a general agreement between the results: fibres with a high, acid labile ATPase reacted with the antisera.

Classification based on the occurrence of the metabolic enzymes was more hazardous. SDH activity was an inconvenient criterion for the initial distinction of fibre types because it showed a gradual transition from low to high. As, however, in the type 3 fibres a considerable range of SDH activity was seen, a subdivision in a type 3^a with a low or intermediate SDH activity and a type 3^b with a high SDH activity was made. There was an overlap in the occurrence of a high SDH activity, indicating aerobic metabolism and in the occurrence of a high LDH activity, indicating anaerobic metabolism. This was seen in part of the red fibres with low ATPase activity (type 4^b) and in the pink fibres (type 2). Johnston *et al.* (1977) found for the LDH activities of pink, white and red body muscle of fish a ratio of 5:3:2. Higher LDH activities in the pink and white fibres than in most red body muscle fibres were also found in this study. However, differences between pink and white fibres were not clearly shown.

The results with the antisera and the results obtained with the ATP-ase reactions were similar for the majority of muscle fibres, but in

those muscle parts showing a mosaic of different fibre types one or two "pink" fibres, not reacting with the antisera and a few "red" fibres of type "3^b" and "4^b" that did react with the antisera were observed. GUTH & YELLIN (1971) found that under the influence of functional demands changes occurred not only in the metabolic properties of muscle fibres, but also in their ATPase activity. During the development of fish muscle fibres the properties of their ATPase change. Those fibres developing into white fibres also acquire other antigenic properties (VAN RAAMSDONK *et al.*, in press). In fishes the number of muscle fibres increases during the whole life (GREER-WALKER, 1970). Our aberrant fibres may represent such changing fibres. Their occurrence is consistent with the above cited literature.

BARANY (1967) showed that the myofibrillar ATPase activity of muscles is correlated with their speed of contraction. Histochemical determination of ATPase activity gave results that corresponded with the biochemical evidence: fast muscle fibres appeared to have a high, alkali stable, acid labile, ATPase activity; slow fibres had a low acid stable, alkali labile, ATPase activity (GUTH & SAMAHA, 1969; BURKE *et al.*, 1973).

In several cases it is assumed that acid resistant ATPase is intermyofibrillar ATPase (GUTH & YELLIN, 1971; GUTH & SAMAHA, 1972). GUTH (1973) states that with the histochemical reaction for ATPase not only myofibrillar ATPase, but also mitochondrial ATPase is demonstrated.

In our study at high magnification a darkly stained intermyofibrillar network could be observed after incubation for ATPase. This was especially the case in white fibres when their myofibrillar ATPase was inactivated by acid pre-incubation. After acid pre-incubation staining of mitochondria was often seen. In this study therefore the ATPase reaction demonstrated not only the myofibrillar ATPase but also the intermyofibrillar (mitochondrial and sarcoplasmic reticular) ATPase. This raises the question as to what amount this intermyofibrillar ATPase interferes with the ATPase properties of the observed fibre types.

In the mitochondrial rich 4^a and 4^b fibres of the perch a low ATPase activity is observed. After incubation for the mitochondrial enzyme SDH in the pink zone a gradual transition from mitochondria rich to mitochondria deficient fibres is seen. The ATPase reactions show no correspondence with this mitochondria distribution, but reveal a sharp border between the red and the pink fibres. This corresponds with the results of the test with the antisera. It can therefore safely be concluded that the contribution of mitochondrial ATPase to the observed muscle fibre ATPase is insignificant.

After acid pre-incubation also no correlation between the mitochondrial SDH distribution and the ATPase reaction is seen. The mitochondria rich 3^b fibres have a high, acid stable, alkali stable ATPase activity similar to that of the 3^a fibres although the latter have very few mitochondria. Therefore it is improbable that the acid resistant ATPase is mitochondrial ATPase. Assuming that most of the acid resistant ATPase is sarcoplasmic reticular, this would imply an enormous difference in sarcoplasmic reticular ATPase between the type 3 fibres and the other fibre types. This seems a most improbable explanation.

A high, alkali stable, acid stable ATPase activity was found by histochemical procedure in the slow contracting embryonic muscle fibres of mammals by GUTH & SAMAHA (1972). Their biochemical ATPase determination, revealing in these fibres a low actomyosin ATPase activity, did not, however, agree with their histochemical data. SAMAHA (1972) showed that in mammalian heart muscle similar discrepancies in histochemical and biochemical data were due to protection of the catalytic site of the myosin ATPase by other muscle proteins.

In this investigation, a high, alkali stable, acid stable ATPase was also found in the 3^a and 3^b fibres of the perch using the histochemical method. These fibres do not react with antisera against white (= fast) muscle proteins. They form the main component of the red part of the levator operculi anterior, a muscle part responsible for lowering the lower jaw during the respiratory movement.

Jaw movements in which the white muscle part also shows EMG activity are at least three times faster than the respiratory movement (OSSE, 1969, p. 350, Fig. 16). In conclusion it can be stated that the high ATPase activity found in these red fibres cannot be correlated with fast contraction; apparently they are slow.

Alkali stable, acid stable ATPase was also found in embryonic fish muscle fibres. These fibres had a low SDH activity and a high LDH activity (VAN RAAMSDONK *et al.*, in press). Of the fibre types described in this study the 3^a fibres with their high, alkali stable, acid stable ATPase activity and their low SDH activity may be differentiating fibres. The gradual increase in SDH activity in these fibres certainly suggests a differentiation. A group of small fibres characterized by a paucity of the enzymes of both anaerobic and aerobic metabolism was also observed in other fishes. It was suggested that these fibres represented a zone of continuous growth (PATTERSON *et al.*, 1975). But a further investigation of these fibres is necessary before any definite conclusions can be made.

The type 3^b fibres have, together with an alkali stable, acid stable ATPase, a high SDH activity. It is very improbable that the red part

of the levator operculi anterior of an approximately 20 cm. long fish would consist mainly of differentiating fibres. These fibres are, therefore, considered to be a red fibre variant. A more extensive investigation of the metabolic properties of red head muscle fibres with this type of high ATPase activity revealed a correlation of these properties with the known characteristics of the red fibres of the body muscles (BARENDS, in preparation).

In muscle fibres of adult mammals three main fibre types are distinguished: fast anaerobic fibres, fast aerobic fibres and slow aerobic fibres (PETER *et al.*, 1972; BURKE *et al.*, 1973). In the perch white fibres show the characteristics of fast anaerobic muscle, the red 4^a fibres those of slow aerobic muscle. The red type 4^b fibres are slow fibres with the enzymes of both aerobic and anaerobic metabolism. The pink fibres are fast fibres which also have the enzymes of both aerobic and anaerobic metabolism. They show the best resemblance to the mammalian fast aerobic fibres. The red fibres with high ATPase (type 3) certainly do not classify for this role. BODDEKE *et al.* (1959) described the presence of white muscle fibres in the red body muscles of the perch. They related the relatively small amount of red muscle to the mode of life of this fish. The perch, a sprinter, showing mainly short bursts of activity when chasing its prey or fleeing from enemies to a hiding place, will rely more on white muscle fibres than on red ones (BODDEKE *et al.*, *op. cit.*). As shown in the present study these scattered "white" fibres in the red zone belong to the pink type.

In the body muscles of fishes at slow swimming speeds only red muscle is used. At high speeds or during bursts of activity EMG activity was recorded from all muscle layers (HUDSON, 1973; JOHNSTON *et al.*, 1977). Therefore, it was expected that in the head muscles red fibres would be used for gill ventilation, a slow continuous activity, and white fibres for the catching of prey. The results obtained by BARENDS (in preparation) in his study on the adductor mandibulae of the perch support this expectation.

In the present study the sternohyoideus was shown to consist of white fibres. In the perch this muscle is known to be only active during rapid vigorous movements such as eating, coughing and gaping (OSSE, 1969). The white parts of the levator operculi anterior and the levator operculi posterior, containing mainly white and pink fibres, were only used during activities of this type and during carbon dioxide induced extreme hyperventilation. The red parts of the levator operculi anterior and of the adductor operculi were also used during respiration (Table 2). So the present study also shows that quick vigorous activity is carried out by a white muscle part and that for a slow sustained activity a red muscle part is used.

The red part of the levator operculi anterior was only used during more intensive respiration. During very quiet respiration no activity was recorded from this muscle. In these instances hardly any jaw movements occur. The rostral bundles of the levator operculi posterior, containing mainly pink and white fibres, were only active during rapid vigorous movements. The middle part of this muscle was a mosaic of red and white fibres and the very small caudal red bundle of this muscle was not accessible without doing a lot of damage. It is therefore not yet possible to obtain reliable EMGs from the red fibres of this muscle. From the red part of the adductor operculi however EMG activity was recorded even during very quiet respiration. It is a general phenomenon that the adductor muscles of the respiratory pump of fishes show continuous activity, whereas the abduction movements are considered to be the results of elastic forces at these low amplitude movements (Osse, 1969, p. 345). In the red part of the adductor operculi and in the red part of the levator operculi anterior the same fibre types: high LDH red fibres (4^b), "high ATPase" red fibres (3^b) and a few 3^a fibres were found. But in the adductor operculi the proportion of 4^b fibres was much greater than in the levator operculi anterior (Figs. 22 and 23). It is probable that the difference observed in the activity of these muscles is correlated with this difference in fibre composition. The possibility, however, that the absence of activity in the levator operculi anterior during very quiet respiration is caused by discomfort as a result of the removal of the skin cannot be entirely excluded.

CONCLUSIONS

a. In the perch 4 main fibre types can be distinguished, 2 types can be classified as white fibres, 2 types are considered to be red types. In each red type two subtypes can be discerned, one of these is possibly a differentiating fibre.

b. The red fibre type showing the best agreement with the accepted characteristics of fish red muscle fibres only occurs in the body muscles. All other fibre types occur both in head and body muscles.

c. Histochemical determination of high, alkali stable ATPase activity in muscle fibres does not imply fast contraction (*c.f.* Guth & Samaha, 1972). It is necessary to determine at least the acid stability of the ATPase activity as well.

d. White and pink fibres of the head muscles are only active during fast movements (eating, coughing, gaping). Red fibres are also active during respiration.

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Ultrastructure of muscle fibres in head and axial muscles of the perch (*Perca fluviatilis L.*)

A quantitative study

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Summary. White, pink, red and deep red fibres, selected from a head muscle and from axial muscles of the perch, show significant differences in actin filament length, Z line thickness, Z line lattice space, myofibril girth, the percentages volume occupied by T system and terminal cisternae of the SR, and in the degree of T system/SR contact per sarcomere. In both muscles the degree of T system/SR contact decreases in the order: white, pink, red, deep red, which suggests a decrease of contraction velocity in the same order.

The position of the T system (at the Z line or at the A/I junction) is related to the actin filament length. The actin filaments in the red fibres are appreciably longer than in the white, which suggests that the sarcomeres of the red fibres have a broader length-tension curve. The Z line thickness is positively correlated with the actin filament length and, in the white and the red fibres, negatively with the degree of sarcomere shortening. Thicker Z lines are suggested to allow greater sarcomere sizes (length or girth).

The percentage volume occupied by mitochondria varies independently of the extent of membrane systems.

The ultrastructural characteristics of the fibre types are in agreement with the functional roles as reported in literature.

Key words: Fish – muscle fibre types – Ultrastructure – Morphometry – Function

Fishes are known to have three main types of muscle fibres, red, pink (or intermediate) and white. Red fibres have high activities in enzymes of the aerobic

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metabolism and a low myosin ATPase activity; they are used during slow sustained movements. White fibres are active during rapid movements; they have a high myosin ATPase activity and show low activities in enzymes of the aerobic metabolism (Bone 1966; Hudson 1973; Johnston et al. 1977; Akster and Osse 1978; Bone et al. 1978; Brill and Dizon 1979). Pink fibres are recruited in movements of intermediate speed; They have intermediate activities in enzymes of the aerobic metabolism and, biochemically, intermediate activity in myosin ATPase (Johnston et al 1977).

Histochemically, pink fibres stain as dark as white fibres with a myofibrillar ATPase method, but they have a greater resistance to extreme pH's (Johnston et al. 1975; Mosse and Hudson 1977; van Raamsdonk et al. 1980). The histochemical ATPase method is useful for distinguishing fibre types, but in fishes the staining intensity is strongly influenced by the temperature stability and pH stability of the enzyme; as a result reliable information on differences in ATPase activity, and thus indirectly on differences in contraction velocity (Barany 1967), cannot easily be obtained (Korneliussen et al. 1978; Akster and Osse 1978; Barends 1979).

Quantitative ultrastructural data concerning Z line thickness, mitochondria and membrane systems have been presented for red and white fibres of fishes (Patterson and Goldspink 1972; Nag 1972; Kilarski 1973) and also for pink fibres of hagfishes and sharks (Korneliussen and Nicolaysen 1975; Kryvi 1977; Slinde and Kryvi 1980).

In addition, some other less common types of fibres have also been described (Patterson et al. 1975; Johnston et al. 1974; Korneliussen et al. 1978; Bone 1978a; Mosse and Hudson 1978; van Raamsdonk et al. 1980). Generally, however, information on this subject is still sparse.

Akster and Osse (1978) have distinguished in the head and the axial muscles of the perch, a fourth fibre type that stains distinctly with the histochemical ATPase reaction, even after preincubation at high or low pH, but does not show any reaction with antisera against white muscle proteins. Both in white and pink fibres a reaction has been obtained with these antisera, but not in red fibres. Succinic dehydrogenase (SDH) activity in this fourth type is highly variable. These investigators have called this type "high ATPase red", but as this name has functional implications which are not justifiable, they will be called "deep red" in the present study, since they are located deep in the red zone of the axial muscles.

In the present study fibres have been selected from a head muscle and from the axial muscles for two reasons. Firstly, all fibre types could be identified in one of these muscles on the basis of topographic criteria, and secondly, this offered an opportunity to compare the ultrastructure of fibres from different parts of the body.

Materials and methods

Perch (*Perca fluviatilis* L.) were caught in the Lauwerszee and kept in tanks (90 × 60 × 40 cm) at 15° C. for 3–6 months. Three specimens, measuring 19–25 cm, were anaesthetised with 0.1 % MS 222 (Sandoz). Shortening of the head muscle, the m. levator operculi anterior, was prevented by immobilizing the jaws in closed position. Fixation was carried out by perfusion with Karnovsky's (1965) fixative, preceded by administering a mixture of 3 parts Hanks balanced salt solution (Difco) and 1 part 0.1 M cacodylatebuffer pH 7.3 containing 10 IU heparin per ml. After perfusion, the m levator operculi anterior and a piece of axial muscle from a place at the level of the second dorsal fin, containing both red

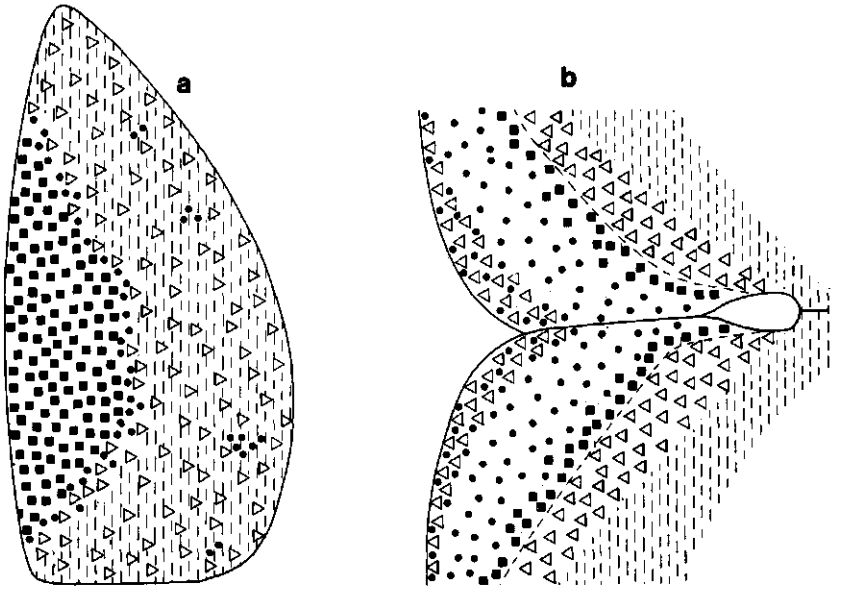


Fig. 1. a Distribution of muscle fibre types in the m. levator operculi anterior. **b** Fibre types in the axial muscles. Symbols: |: white fibres; △: pink fibres; ●: red fibres; ■: deep red fibres

and white fibres, were excised and left overnight in Karnovsky's fixative. The red and white parts were then separated, cut into small pieces, postfixed in 2% OsO_4, dehydrated, and embedded in an Epon mixture. Muscle fibres of the four types were selected in 1 µm sections stained with p-phenylene-diamine (Korneliussen 1972).

M. levator operculi

White, pink and red fibres were selected in transverse sections from the white part of this muscle (Fig. 1a). Fibres with many subsarcolemmal and intermyofibrillar mitochondria were considered as red fibres. Fibres with scarce subsarcolemmal mitochondria and with an intermediate number of intermyofibrillar mitochondria were considered as pink fibres. White fibres were selected on the basis of the scarcity of mitochondria throughout the fibre (Fig. 3a). Deep red fibres were taken from the centre of the red part of this muscle which contains only this type (Figs. 1a, 3b).

After cutting ultrathin transverse sections of the head muscle fibres, the Epon blocks were readjusted for cutting longitudinal sections.

Axial muscles

White fibres from the axial muscles were taken from deep in the white zone, well away from areas containing pink fibres (Fig. 1b). Pink, red and deep red fibres were selected from longitudinal sections as indicated in Fig. 4. The most lateral fibre layers from the red zone were not used, as in this part pink fibres are found amongst the red fibres (Fig. 1b). As head muscles could be fixed more accurately at rest length than axial muscles, the former were studied more extensively.

Measurements

Cross sections of whole fibres were photographed a low magnification (×4,800). From each fibre 3 consecutive micrographs (magnification ×27,600, total area 128 µm^2) were taken from transverse sections (see Fig. 5) and 3 separate micrographs (magnification ×35,000, total area 80 µm^2) were taken from longitudinal sections. The areas to be photographed were selected at a magnification that does not allow distinction of T tubules. Volume percentages were measured by a point counting method. The line length

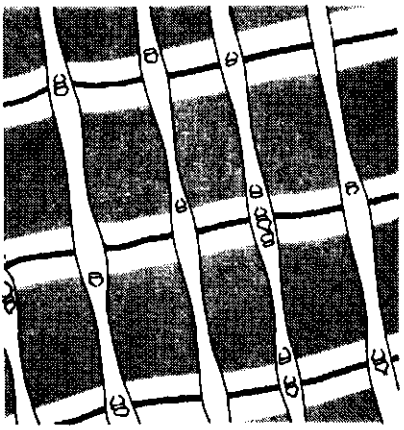


Fig. 2. Measurement of the encircling of the sarcomeres by T tubule and by the degree of T system/SR contact per sarcomere. If the T system surrounds the whole sarcomere at Z line level (maximal encircling = 1) or at both A/I junctions (maximal encircling = 2), a T tubule profile will be found at every Z line or A/I junction. The sample area contains 12 sites at the Z line and 24 sites at the A/I junction. 6 of the 12 sites at the Z line are occupied by a T tubule profile. Encircling of the sarcomeres by T tubule at the Z line = $6/12 \times 1 = 0.5$, one T tubule profile forms a diad, 5 form triads; encircling of the sarcomere by diads at the Z line is $0.5 + 5/6 \times 0.5 = 0.92$. 8 of the 24 sites at the A/I junction are occupied by a T tubule. Encircling of the sarcomeres by T tubule at the A/I junction = $8/24 \times 2 = 0.67$. 7 T tubule profiles form diads, one forms a triad. Encircling of the sarcomere by diads at the A/I junction is $0.67 + 1/8 \times 0.67 = 0.75$. Total encircling of the sarcomeres by T tubule in this fibre = $0.5 + 0.67 = 1.17$. Total encircling of the sarcomeres by diads in this fibre = $0.92 + 0.75 = 1.67$

of the intermyofibrillar space per unit cross section area was measured by counting the number of intersections with the lines of a test grid (Weibel 1973; Eisenberg et al. 1974).

Sarcomere components. Sarcomere length, myosin filament length, actin filament length (Fig. 9a) and Z line thickness were measured, using vernier callipers or an ocular micrometer, in micrographs of longitudinal sections. Z line thickness was measured irrespective of the shape of the Z line profile. In each micrograph five Z lines were selected at random. The points where the actin filaments appear to thicken upon entering the Z line were chosen as the limits of the Z line. Z line lattice space was measured in micrographs of transverse sections in the border zone of Z line and I band; the line length of the intermyofibrillar space, a measure for sarcomere girth, was measured with a grid line distance of 0.47 μm, also in transverse sections.

Membrane systems. The following parameters were measured in micrographs of longitudinal sections:

1. The percentage volume occupied by the terminal cisternae of the sarcoplasmic reticulum (SR), expressed as percentage of the myofibrillar volume of the fibres. This percentage is corrected for differences in the volume percentage of mitochondria between the fibre types. The test grid, oriented at an angle of 19°/71° to the sarcomere axis (Sitte 1967), had a line distance of 0.14 μm.

2. The percentage volume occupied by the T system, is also expressed as percentage of the myofibrillar volume of the fibres and measured with the same test grid.

3. The degree to which the sarcomere outline is encircled by T tubule (encircling by T system) (Fig. 2).

4. The degree of diadic contact between the T system and the terminal cisternae of the SR per sarcomere (T system/SR contact). This was calculated from data obtained under 3 by counting one triad as two diads (Fig. 2). Parameters 2 and 3 cover only T tubules that are part of a triad or diad, since T tubules without contact with the SR cannot always be recognized.

The percentage volume occupied by the entire SR has not been measured, as the required additional oblique sections (Weibel 1972) could not be prepared from the same fibres.

Myofibrils and mitochondria. Fibre cross-section area and the percentages volume occupied by the myofibrillar core and the mitochondria outside this core (Fig. 5) were measured in micrographs of whole fibres with a grid line distance of 1 µm. The percentages volume occupied by the intermyofibrillar mitochondria and the rest of the intermyofibrillar space were measured in micrographs of transverse sections with a grid line distance of 0.24 µm. These measurements served for calculating the total percentages of volume occupied by mitochondria and myofibrils per fibre.

Statistics

The significance of the differences between the fibre types of the head muscle were tested with Student's *t*-test. Red and deep red fibres, neither of which reacted with antisera against white muscle proteins, have been put together into a combined red group, the white and pink fibres into a combined white group. The significance of the differences between the two combined groups, between white and pink, and between red and deep red fibres have been tested. For each fibre type the significance of the differences between head and axial muscle fibres has also been tested, with Student's *t*-test.

Correlation coefficients and their significance ($P<0.05$) were calculated according to Sokal and Rohlf (1969).

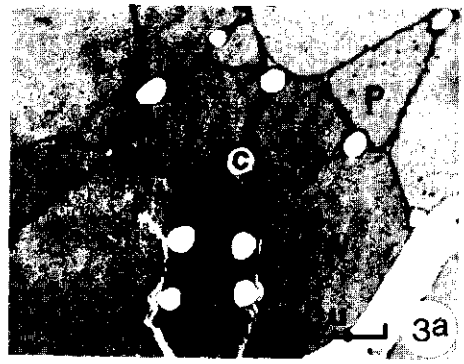
Results

General observations

There are striking differences in vascularization between different types of fibres, as previously noticed by Boddeke et al. (1959), Bone (1978a), Mosse (1978, 1979) and Barends (1979). Red and deep red fibres, even those present in a white muscle part, have more capillaries per fibre area than white and pink fibres; pink fibres have more capillaries than white fibres (Figs. 3a, b, 4).

In the large white and pink fibres of the head muscle, small polygonal myofibrils are present in the whole fibre. Fibres of both red types and the smaller pink and white fibres have an outer layer of ribbon-like myofibrils, while the more centrally located myofibrils are small and polygonal (Figs. 5, 6). In all types the sarcomeres are not entirely in register (Figs. 5, 6). In nearly all muscle fibres, the sarcomeres show a distinct banding pattern with straight Z lines and distinct M bands. Some of the fibres from the red part of the m. levator operculi anterior in specimen 1 show irregular, wayy A/I band borders and Z line streaming, which are known to occur mainly in hypertrophying red muscle fibres (Morton 1973). In sections stained with p-phenylenediamine, these fibres can be distinguished from the surrounding deep red fibres by the lighter background staining; they were set apart as a separate group of aberrant fibres.

In fibres of different types, remarkable differences can be noticed in the position of the T system. In white and pink fibres and in the red fibres of the axial muscles, the T system is found next to the Z line, in the deep red fibres this system is mainly found next to the A/I band junction. In the red fibres of the m. levator operculi the T system can be observed in both locations, even in one and the same muscle fibre (Figs. 7–9). T system/SR contacts at the Z line are mainly triads, at the A/I junction mainly diads (Fig. 16). The longitudinal tubuli of the SR are more slender in deep red fibres than in the other types. Red and deep red fibres contain more ribosomes and more glycogen granules than white and pink fibres, but there are individual differences in the glycogen content between the specimens.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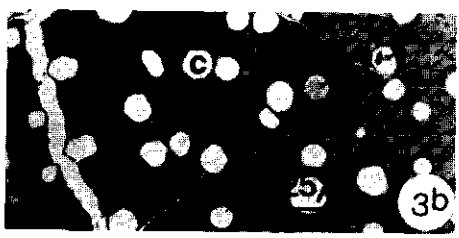
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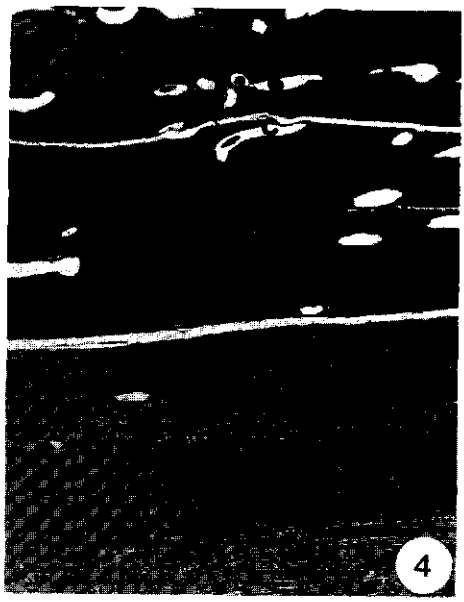
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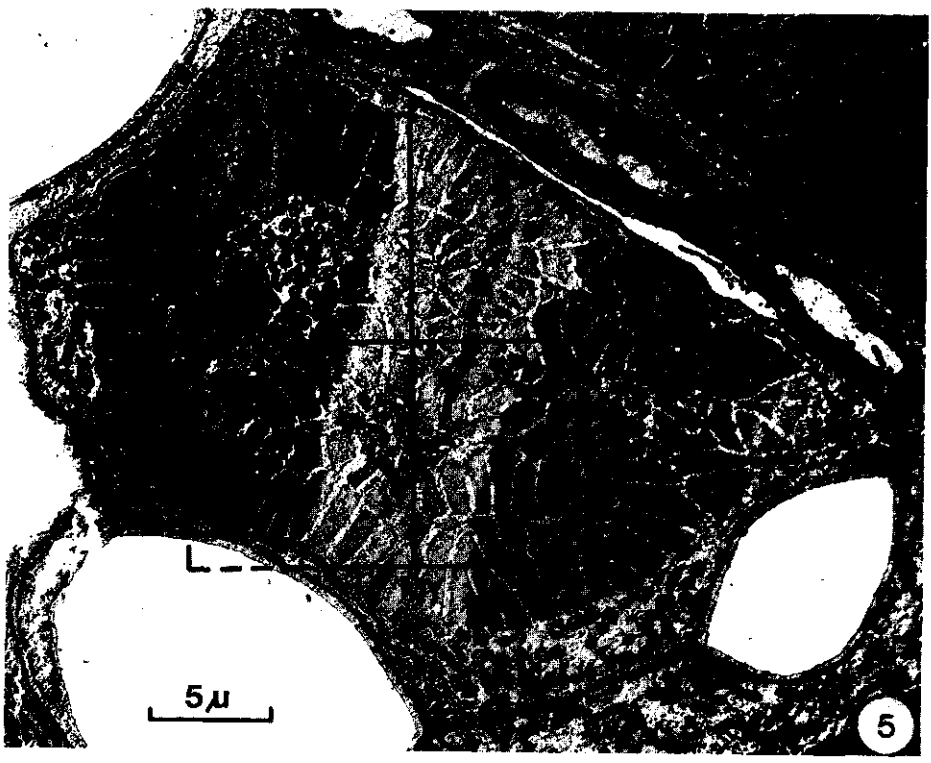
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Quantitative data

Sarcomere components (Fig. 10). In the head muscles, the white fibres have the shortest sarcomeres, the shortest actin filaments, and the thinnest Z lines. The values for these parameters increase in the order pink, red, deep red. The differences between the two combined groups are highly significant ($P < 0.001$). Red fibres are

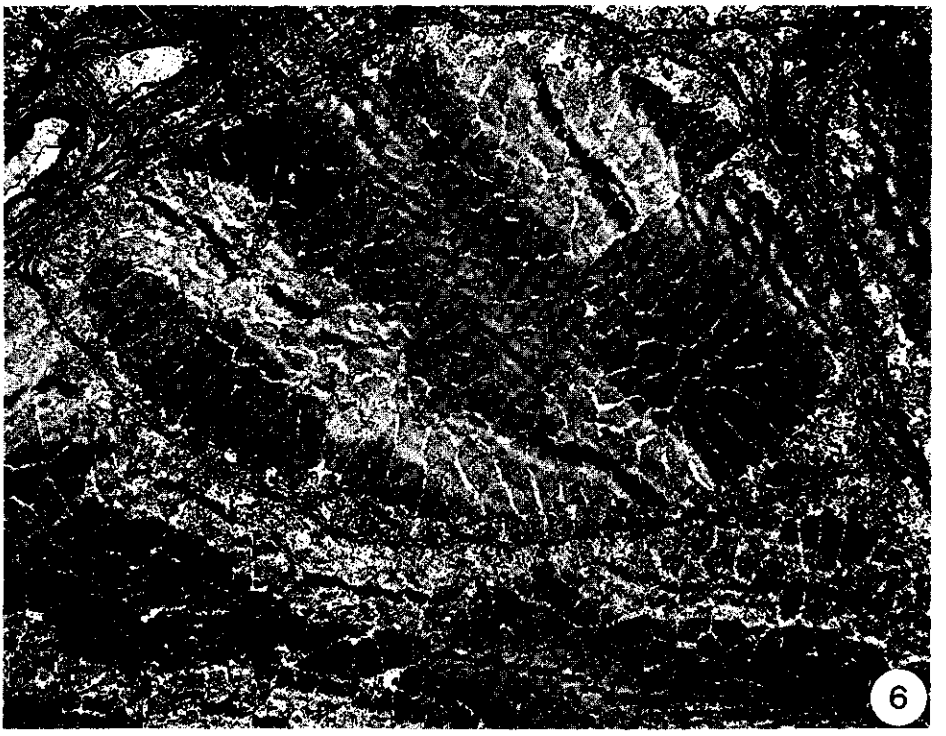

Fig. 3a, b. System of fibres from the white part (**a**) and from the red part (**b**) of the m. levator operculi anterior. Note the many capillaries, dilated by perfusion with heparin, that surround the red fibres in both muscle parts, whereas only a few are present around the white fibres. Red fibres and deep red fibres with many mitochondria, visible as dark subsarcolemmal aggregates and as dark dots between the myofibrils. White fibres are poor in mitochondria. Pink fibres contain more mitochondria than white fibres but less than red fibres. *R* red fibre, *P* pink fibre, *W* white fibre, *c* capillary

Fig. 4. Body muscles; longitudinal section of the red zone and the pink zone. Note the difference in capillaries and in mitochondria (visible as dark spots). Pink fibres were selected from the pink zone close to the red zone. Small fibres, relatively poor in mitochondria, in the red zone close to the pink zone were selected as deep red fibres. More lateral fibres rich in mitochondria are red fibres. *c* Capillary, *DR* deep red fibre, *P* pink fibre, *R* red fibre

Fig. 5. Micrograph of a red fibre of the head muscle. Note the myofibrillar core (*My*), the subsarcolemmal mitochondria (*SM*), and the intermyofibrillar mitochondria (*IM*). Three micrographs of medium magnification were made of the myofibrillar core, as indicated. Red fibres and small white fibres (Fig. 6) have an outer row of ribbonshaped myofibrils

Fig. 6. White fibres of the head muscle. In large white fibres the outer myofibrils are small and polygonal. Several sarcomere bands are seen, caused by the sarcomeres not being perfectly in register

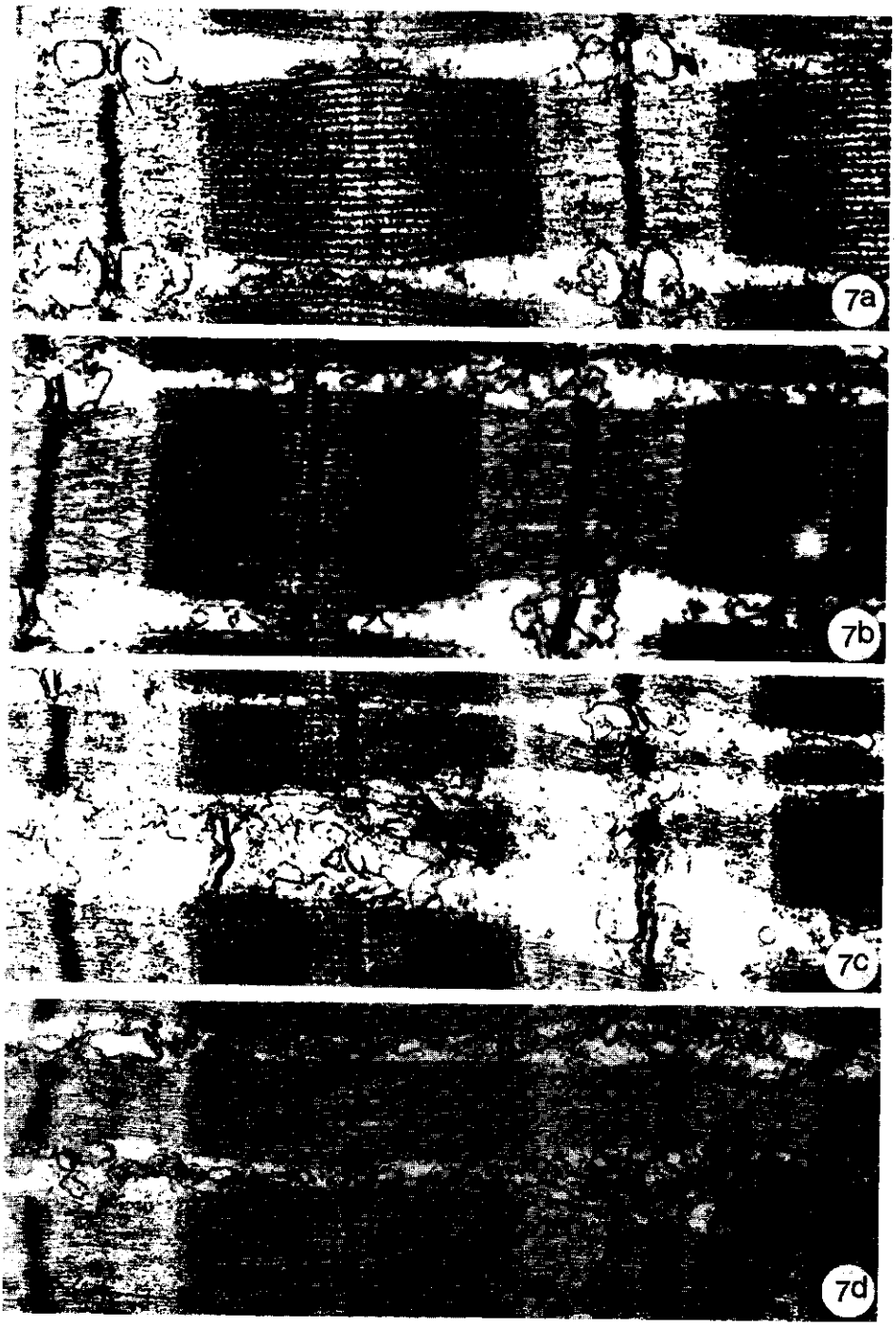

Fig. 7a–d. Sarcomeres of white (**a**), pink (**b**), red (**c**) and deep red (**d**) fibres of the head muscle. Note the positions of the T system (*arrows*) and the large terminal cisternae in the white fibre

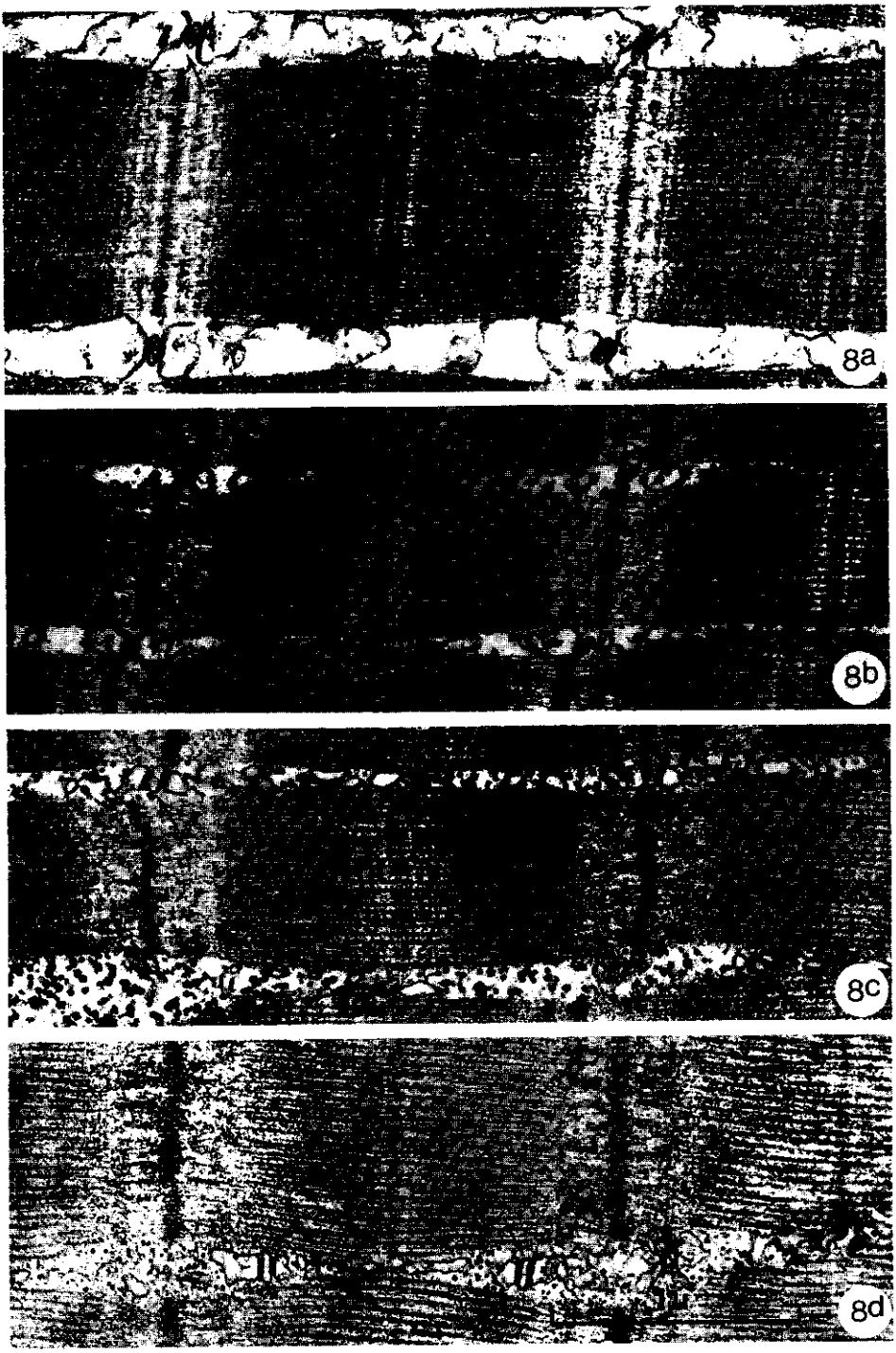

Fig. 8a–d. Sarcomeres of white (**a**), pink (**b**), red (**c**), and deep red (**d**) fibres of the axial muscles. Note the positions of the T system (*arrows*), the large terminal cisternae in the white fibre, and that the sarcomeres are shorter than those of the head muscle

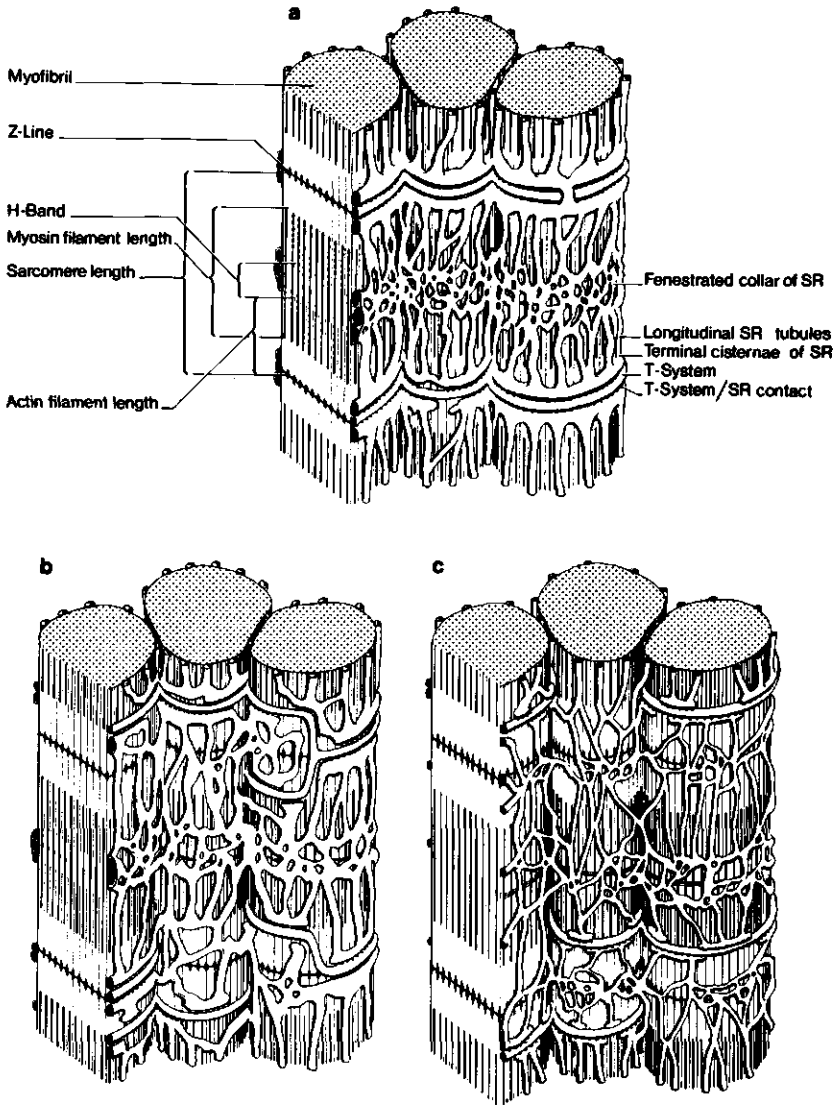

Fig. 9a–c. Schematic representation of the sarcomere components and of the membrane systems. **a** White fibre, the T system is found at the Z line. **b** Red fibre of the head muscle, the T system can be observed at the Z line as well as at the A/I junction. **c** Deep red fibre, the T system is located at the A/I junction. The distribution of the T system and the T system/SR contact is more irregular in red and deep red fibres

significantly different from deep red fibres in sarcomere length ($P < 0.01$) and in Z line thickness ($P < 0.001$). The white and pink fibres are significantly different in Z line thickness ($P < 0.05$) (Fig. 10a, b, e).

The fibre types of the axial muscles show a similar range of sarcomere length, actin filament length and Z line thickness as the fibres of the head muscle, but in the

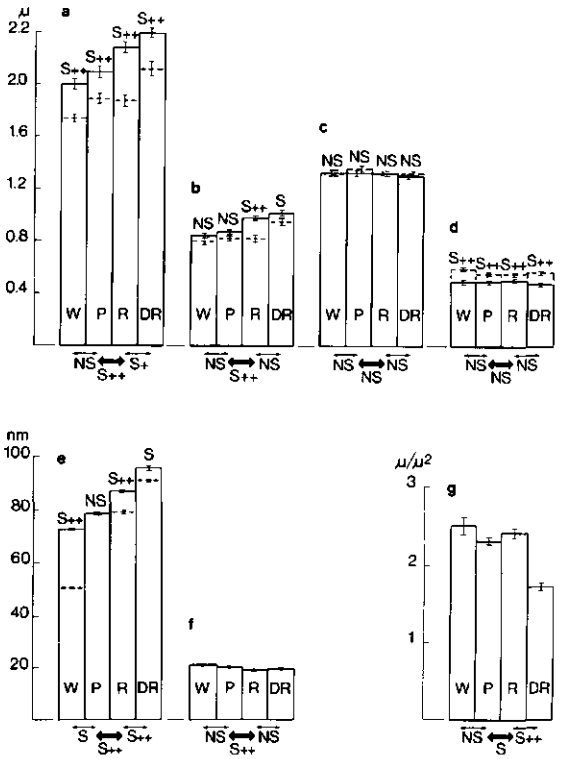

Fig. 10a–g. Sarcomere components, mean values and standard error of mean, axial muscles dotted. **a** Sarcomere length; **b** actin filament length; **c** myosin filament length; **d** overlap of actin and myosin; **e** Z line thickness; **f** Z line lattice space; **g** line length of intermyofibrillar space. *W*, white fibres, head: $n = 15$, axial: $n = 14$; *P*, pink fibres, head: $n = 15$, axial: $n = 11$; *R*, red fibres, head: $n = 19$, axial: $n = 16$; *DR*, deep red fibres, head: $n = 20$, axial: $n = 12$. Significance of difference between head and body muscle fibres on top of each column. Significance of difference between head muscle fibres types below the graph. ↔ : significance of difference between white and pink fibres or between red and deep red fibres. ⟷: significance of difference between the combined white and pink fibres and the combined red and deep red fibres. NS, not significant; S, $P < 0.05$; S+, $P < 0.01$; S++, $P < 0.001$

red axial muscles, actin filament length and Z line thickness have the same values as in the pink fibres of both muscles.

Deep red axial muscle has shorter actin filaments than deep red head muscle ($P < 0.05$). In the white and in the pink fibres of the two muscles, the actin filament lengths are not significantly different. The Z line thickness of the pink fibres is not significantly different in the two muscles. In the other three fibre types, the axial muscles have thinner Z lines than the head muscles ($P < 0.05 - P < 0.001$). All fibre types of the axial muscles are more shortened than the fibres of the head muscle; this is apparent from the greater overlap of actin and myosin filaments ($P < 0.001$). The differences in shortening for the head muscle types are not significant (Fig. 10d).

The myosin filament length is similar in all the studied groups (Fig. 10c).

The fibre types of the head muscle show also differences in Z line lattice space and in myofibril girth; the pink and white fibres have significantly ($P < 0.05$) larger

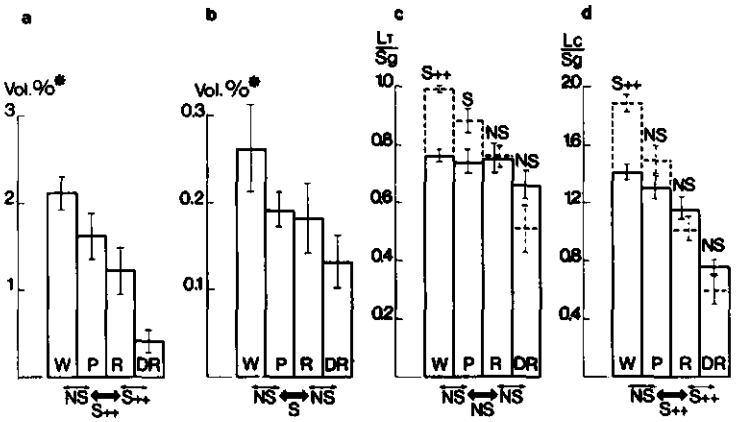

Fig. 11a–d. Membrane components, mean values and standard error of mean, body muscles dotted. **a** Terminal cisternae of SR; **b** T tubule; **c** encircling by T system; T tubule length (LT)/sarcomere girth (Sg); **d** T system/SR contact. Length of the diadic contact (Lc)/sarcomere girth (Sg). * percentage of myofibrillar volume; note the different scales. *W* white fibres: *P* pink fibres; *R* red fibres; *DR* deep red fibres. For sample sizes and explanation of significance symbols see Fig. 10

Z line lattice spaces than the two red fibre types (Fig. 10f). In the deep red fibres the line length of the intermyofibrillar space is smaller than in the other types. The difference between the red and deep red fibres is highly significant ($P < 0.001$). This means that the deep red fibres have a greater myofibril girth than the other fibre types (Fig. 10g).

Membrane systems. The highest values for the percentage volume occupied by the T system, the percentage volume occupied by the terminal cisternae of the SR, and for the degree in which the sarcomeres are encircled by T system/SR contact, have been found in the white fibres; the values decrease for pink, red, and deep red fibres (Fig. 11a, b, d). In the head muscle, the percentage volume occupied by terminal cisternae of the SR and the degree of T system/SR contact per sarcomere are significantly different ($P < 0.001$) for the two combined groups and also for red and deep red fibres. The white fibres of the axial muscles have significantly ($P < 0.001$) more T system/SR contact than the white fibres of the head muscle. In the other fibre types, the differences in this parameter are not significant between the two muscles (Fig. 11d). The percentage volume occupied by the T system is significantly different in the two combined groups ($P < 0.05$) (Fig. 11b).

In the encircling of the sarcomeres by T system, no significant differences have been found between the fibre types of the head muscle. In the axial muscles this parameter shows also a gradual change in the fibre types; it is significantly different for both muscles in the pink fibres ($P < 0.05$) and in the white fibres ($P < 0.001$) (Fig. 11c).

Mitochondria and myofibrils. These were only measured in fibres of the head muscle. The white fibres are not significantly thicker than the pink fibres. Both types of red fibres are much thinner. There is no difference in the fibre cross section

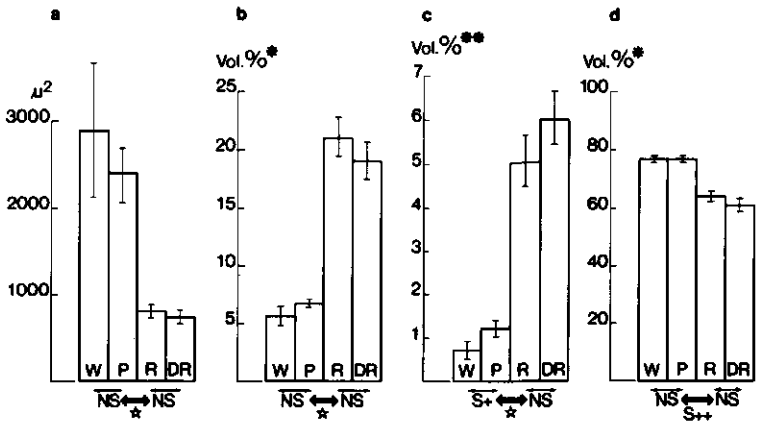

Fig. 12a–d. Composition of head muscle fibres, mean values and standard error of mean. **a** Fibre cross section area; **b** total mitochondria; **c** intermyofibrillar mitochondria; **d** myofibrils. * percentage of fibre volume; ** percentage of volume of myofibrillar core. *W* white fibres, $n=13$; *P* pink fibres, $n=11$; *R* red fibres, $n=15$; *DR* deep red fibres, $n=20$; ☆ striking difference, test of significance not applied. For explanation of the other significance symbols, see Fig. 10

area of red and deep red fibres of the head muscle (Fig. 12a). As expected, the two combined groups show considerable differences in the percentages volume occupied by mitochondria and consequently also in the percentage volume occupied by myofibrils. White and pink fibres are significantly different ($P<0.05$) in the percentage volume occupied by intermyofibrillar mitochondria, but not in the total percentage volume occupied by mitochondria or in the percentages volume occupied by myofibrils. Red and deep red fibres of the head muscle differ not significantly in these parameters (Fig. 12b, c, d).

Aberrant fibres. The aberrant fibres of the red zone of the m. levator operculi of one of the fishes had a Z line thickness of 73 nm., similar to that of the pink fibres, and a sarcomere length of 2.3 μ; this value is intermediate between those for the red and the deep red fibres.

Discussion

For a better understanding of the meaning of the differences between the fibre types, the parameters will first be discussed separately.

Mitochondria and myofibrils

The area occupied by mitochondria, and more specifically, by intermyofibrillar mitochondria (Müller 1976), is considered to be related to a muscle fibre's resistance to fatique, the fibre cross-section area and the percentage volume occupied by myofibrils to the contraction force (Close 1972; Burke 1978). These parameters are not significantly different for red and deep red fibres. The differences in the percentages volume occupied by mitochondria between the two combined groups

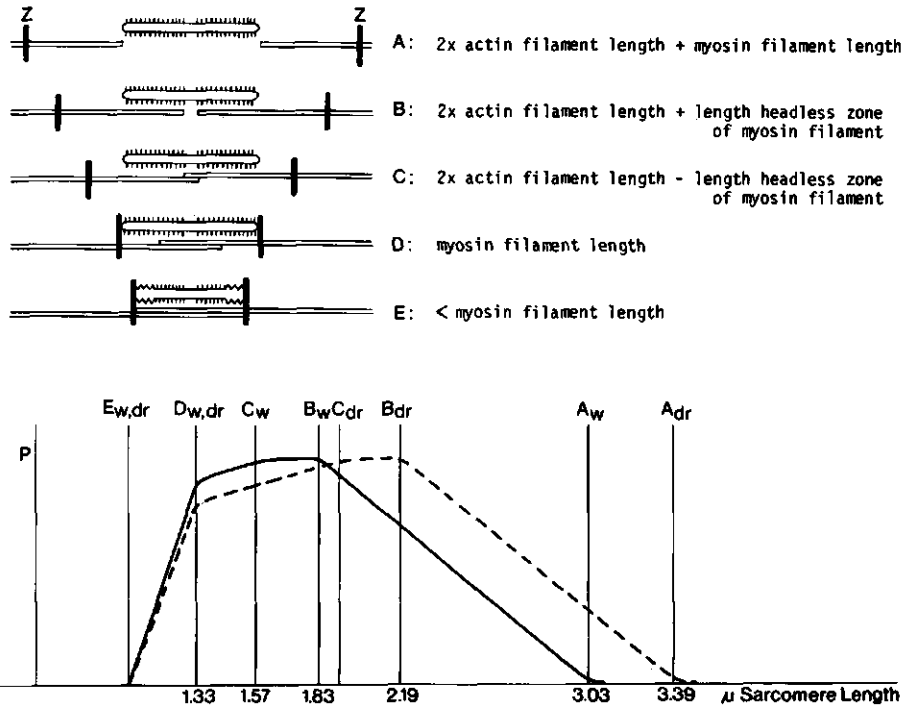

Fig. 13. Predicted shape of length-tension curves for white (w, —) and deep red (dr, ---) sarcomeres (After Gordon, Julian and Huxley 1966). A–E: filament overlaps as indicated in the upper part of the figure

and the difference in the percentages volume occupied by intermyofibrillar mitochondria between the white and the pink head muscle fibres can be attributed to the selection. Although the percentage volume occupied by intermyofibrillar mitochondria is significantly different for white and pink head muscle fibres, a distinction between these types based on this criterion is not accompanied by significant differences in most of the other parameters.

Sarcomere components

Myosin filaments are considered to have a fairly constant natural length of about 1.6 μm (Page and Huxley 1963; Close 1972; Walker and Schrodt 1974). The muscle fibre types of the perch have similar myosin filament lengths. The obtained values (after fixation) are slightly lower than those mentioned for other vertebrates (Walker and Schrodt 1974).

Fibres with long actin filaments are capable of greater excursion than those with short filaments. According to Gordon et al. (1966) and to Walker and Schrodt (1974), an increase of the actin filament length produces a broader length-tension curve. Fig. 13 (modified after Gordon et al. 1966) shows the predicted shape of the length-tension curves for white and deep red fibres (the extremes of the range). The distance C-D, representing that part of the graph where the actin filaments slide into the opposite part of the sarcomere until the Z lines reach the myosin filaments,

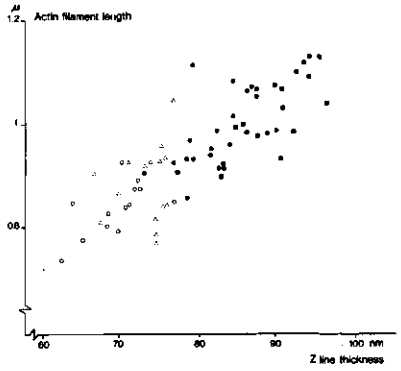

Fig. 14. Scatter-diagram of actin filament lenght and Z line thickness in the m. levator operculi anterior. There is a fair correlation ($r = 0.8$; $n = 68$) between these parameters; o white fibres; △ pink fibres; ● red fibres; ■ deep red fibres

is dependent upon the actin filament length. As this distance is larger in sarcomeres with long actin filaments, these will have broader length-tension curves.

The length of the actin filaments also affects the fibre contraction velocity. As fibres with long actin filaments have less sarcomeres in series per unit fibre length than fibres with short actin filaments, the latter must have a greater overall velocity, even if the sarcomere velocity is equal in both fibres (Close 1965).

Although Z line thickness has been described as a useful ultrastructural criterion for distinguishing muscle fibre types in mammals (Tomanek et al. 1973; Eisenberg and Kuda 1976; Van Winkle and Schwartz 1978) and in fishes (Patterson and Goldspink 1972), the functional implications are unknown. Salmons et al. (1978) found in fast fibres of rabbits that were changed into slow fibres an increase in Z line thickness, which suggests an inverse relation between the Z line thickness and the contraction velocity. Eisenberg and Kuda (1976) found in guinea pig muscle a negative correlation for the Z line thickness and the percentages of volume occupied by the T system and the terminal sacs of the SR. In rat muscle this relation was not found, here the combined presence of thick Z lines and many mitochondria suggests a relation between the Z line thickness and the resistance to fatique (Schiaffino et al. 1970).

The muscle fibres of the perch show, from white to deep red, a decrease in the parameters for the membrane components combined with an increase in Z line thickness and the percentage volume occupied by mitochondria. But these relations are not consistent. Red and deep red head muscle fibres are significantly different in Z line thickness, but not in the volume percentages of mitochondria; red head and body muscle fibres that also are significantly different in Z line thickness, are not different in the degree of T system/SR contact. The actin filament length shows a more consistent correlation with the Z line thickness. The difference in Z line thickness between the white fibres of the head muscle and the axial muscles, which have similar actin filament lengths, is probably caused by the greater shortening of the axial muscles. Multiple correlation of the Z line thickness with the actin filament length and the degree of overlap between actin and myosin filaments produced in each of the fibre types (head and body muscles combined) a significant ($P < 0.05$) correlation of the Z line thickness and the actin filament length (white, $r = 0.7$; pink, $r = 0.4$; red, $r = 0.6$; deep red, $r = 0.6$) and in the white and the red fibres a

significant negative correlation of Z line thickness and the overlap of actin and myosin (r resp. –0.8 and –0.4).

During shortening the sarcomeres expand laterally, and this is accompanied by an increase in A band lattice space (Elliott et al. 1967). Lateral Z line expansion, which is less than A band expansion, is also known (Goldspink 1971; Dragomir and Ionescu 1975). Goldspink (1971) and Patterson and Goldspink (1976) mentioned that during contraction the actin filaments exert an oblique pull on the Z line. They suggested that the obliqueness of this pull, which is partly counteracted by the lateral expansion of the Z line, increases with the myofibril girth and causes growing myofibrils to split upon reaching a critical size.

Sarcomeres behave as constant volume systems (Elliott et al. 1970). Thus maximal shortening of sarcomeres with long actin filaments can be expected to cause a greater A band expansion resulting in increased obliqueness of pull of the actin filaments on the Z line. As the Z line has a zig-zag structure it is conceivable that the thicker Z lines and smaller Z line lattice space, found in fibres with long actin filaments, will allow greater stretching (unfolding) of the Z line during sarcomere shortening. Thus by counteracting the obliqueness of pull of the actin filaments excessive splitting up of myofibrils in these fibres will be prevented. Although the studied fibres were not prepared for measuring the relation between Z line thickness and sarcomere contraction, an indication is found that during sarcomere shortening the Z line decreases in thickness. Patterson and Goldspink (1972, 1976) described in the red fibres of the coalfish, *Gadus virens*, thicker Z lines and a smaller Z line lattice space together with a less oblique pull of the actin filaments on the Z line and a larger myofibril size than in white fibres; a combination of characters which is in agreement with the above hypothesis.

Membrane systems

In studies comparing the ultrastructure of slow and fast fibres, the former generally are found to have less extensive membrane systems and less T system/SR contact than the latter (Page 1968b; Hess 1970; Franzini Armstrong 1973; Schiaffino et al. 1970; Eisenberg and Kuda 1976; Van Winkle and Schwartz 1978). In red axial muscles of fishes also less extensive membrane systems have been measured than in white muscles (Nag 1972; Kilarski 1973; Patterson and Goldspink 1972; Kryvi 1972). Intermediate (pink) fibres of the hagfish, *Myxine glutinosa*, have intermediate extensive membrane systems (Korneliussen and Nicolaysen 1975). Although the described differences are not always significant, they are roughly in accordance with each other and with the data obtained in the present study.

The differences in the degree of T system/SR contact between the fibre types were similar in the head muscle and in the axial muscles of the perch except for the white and the pink fibres of the head muscle, an exception that may be due to some pink fibres accidentally included in the white. This parameter seems to be a reliable criterion for distinguishing muscle fibre types. Besides, being a ratio of line lengths, it is relatively insensitive to the osmotic value of the fixation medium. But in addition to this parameter the size of the sarcomeres should be measured also. The percentage volume occupied by the terminal cisternae of the SR is probably also a useful criterion for distinguishing muscle fibre types. The percentage volume

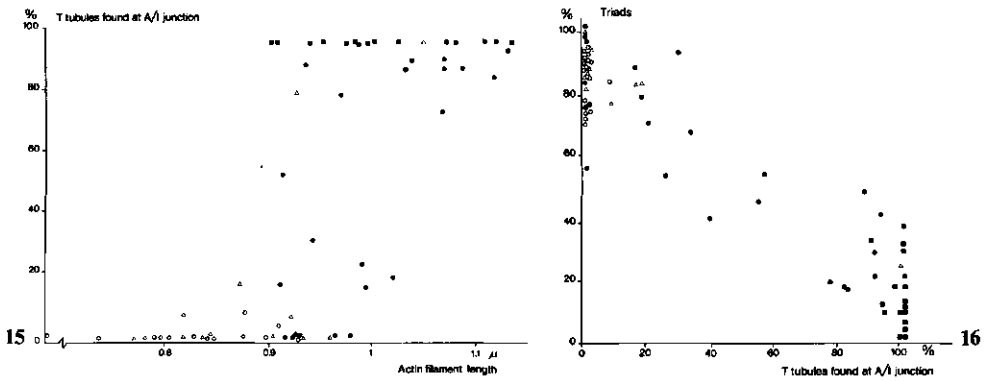

Fig. 15. Scatter-diagram of actin filament length and the position of the T system in the levator operculi posterior. Sarcomeres with short actin filaments have the T system/SR contact at the Z line; in fibres with longer actin filaments, more T system/SR contacts are found at the A/I junction; ○ white fibres; △ pink fibres; ● red fibres; ■ deep red fibres

Fig. 16. Scatter-diagram of the position of the T system and the location of triads and diads in the m. levator operculi anterior; 0% T tubules at the A/I junction means that all T tubules are found at the Z line; 0% triads means that all T system/SR contacts are diads. Most of the T tubule/SR contacts at the Z line are triads, most of these at the A/I junction are diads; ○ white fibres; △ pink fibres; ● red fibres; ■ deep red fibres

occupied by the T system and the degree of encircling of the sarcomeres by T tubule are both very variable and would require large sample sizes.

Position of the T system

Page (1968a, b) found by comparing muscles of various vertebrates that, irrespective of the contraction velocity, in fibres with actin filaments shorter than 25 periods of 38.5 nm (natural length) the T system/SR contacts are generally located at the Z line, while fibres with longer actin filaments have the T system/SR contacts at the A/I junction. Exceptions to this rule are some very fast-acting fibres in which short actin filaments coincide with T system/SR contacts at the A/I junction (Franzini-Armstrong 1973). The actin filament length of white and pink fibres and of the red fibres of the axial muscles of the perch, which have the T system at the Z line, is generally below 0.9 μm (length after fixation). The deep red fibres which have the T system at the A/I junction have actin filament lengths of about 1 μm (Figs. 10b, 15). Location of the T system at the A/I junction is not accompanied by a high degree of T system/SR contact, owing to a more irregular distribution of the T system at the A/I junction than at the Z line, and to the presence of mainly diads at the A/I junction and of triads at the Z line.

According to Winegrad (1970) the efflux of calcium from the SR towards the sarcoplasm at the activation of muscle fibres is mainly from the terminal cisternae, while the reabsorbtion takes place over the whole SR. In the sarcomeres calcium acts on the troponin/tropomyosin complexes, located on the actin filaments (Ebashi and Endo 1968; Ebashi and Nonomura 1973). An increase of the distance between the terminal cisternae and the troponin/tropomyosin complexes in

transverse direction, caused by an increase in myofibril girth, also increases the distance to the calcium absorbing tubular elements of the SR. An increase of this distance in longitudinal direction does not increase the distance to the tubular elements, since these surround the sarcomere over its entire length. The simultaneous uptake and release of calcium during muscle activity, as described by Winegrad (1970), may cause a diminishing calcium gradient along the actin filaments and thus set a limit to the distance between the triads and the tips of these filaments. The position of the T system/SR contact at the A/I junction in fibres with long actin filaments may be a structural adaptation to ensure a sufficient calcium supply to all troponin/tropomyosin complexes. It is remarkable, however, that this limit would be the same for both fast and slow fibres, as it is known that there are differences in the response of myofibrillar ATPase to changing sarcoplasmic calcium concentrations (Perry et al. 1979), in the rate of calcium reabsorbtion by the SR (Sreter 1969; Van Winkle et al. 1978), and in the amount of SR per fibre (Nag 1972; Kilarski 1973; Van Winkle and Schwartz 1978).

Fibre types

The four fibre types described in this paper are significantly different as regards one or more of the morphological parameters. Most of these gradually change in the order white, pink, red, deep red. This confirms the observations of Korneliussen and Nicolaysen (1975), of Johnston et al (1977) and of Slinde and Kryvi (1980), that pink fibres are intermediate between white fibres and red fibres. From the present study it appears that in the perch the deep red fibres occupy an extreme position on the red side.

Histochemical classification of the muscle fibre types based on reactions with specific antisera, and to a lesser extent on ATPase activity, resulted in establishing a group containing white and pink fibres, and a group containing red and deep red fibres (Akster and Osse 1978). Actin filament length and Z line thickness, which have the same values in pink fibres and red fibres of the axial muscles do not match the histochemical classification. The differences in T system/SR contact are better in agreement.

The histochemical reactions mentioned above, as well as the extent of membrane systems, are generally considered as indicative of the contraction velocity. Structural differences related to other functions do not necessarily apply to these types, as appears from the difference in actin filament length (related to the length-tension curves) between the red fibres of both muscles. Succinate dehydrogenase activity (related to the resistance to fatigue) varies between the deep red fibres of both muscles (Akster and Osse 1978). An independent variation of the contraction velocity and the resistance to fatigue has already been described for mammalian muscle (Close 1972; Burke et al. 1973; Burke 1978).

Deriving functional properties from structural data is somewhat speculative, but as yet it is not quite possible to compare the predicted differences in mechanical properties of the fibre types with experimental data. Flitney and Johnston (1979) mentioned for isolated fibres of the m. levator operculi anterior of *Tilapia mossambica*, that white fibres have a greater maximal velocity than red fibres. But data on other muscle fibre types of fishes are not yet available.

Functional role of the fibre types

It may be concluded that the morphology of white, pink and red fibers is in agreement with the function as reported in literature (for a review see Bone 1978b). On the basis of their ultrastructure, deep red fibres are expected to have great excursion lengths, broad length-tension curves, and a low contraction velocity (this in contrast to their dark staining with a histochemical ATPase method). The role of small clusters of deep red fibres in the axial muscles is not clear. The presence of these fibres in head muscles is better understandable. In fishes, an increase in respiration is effected in the first place by an increase in stroke volume. Only at high respiration levels the cycle frequency increases (Hughes and Saunders 1970). During increased ventilation of the perch, coinciding with activity of the deep red fibres of the m. levator operculi anterior (Akster and Osse 1978), no higher cycle frequency was found, but the lower jaw excursion and the opercular abduction were considerably enlarged. The function of the deep red fibres in this movement is in agreement with the morphology.

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A COMPARATIVE STUDY OF FIBRE TYPE CHARACTERISTICS AND TERMINAL INNERVATION IN HEAD AND AXIAL MUSCLE OF THE CARP (*CYPRINUS CARPIO* L.): A HISTOCHEMICAL AND ELECTRON-MICROSCOPICAL STUDY

by

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SUMMARY

In the axial muscles and in a head muscle (m. hyohyoideus) of the carp two slow (red) and three fast (white, large pink and small pink) fibre types were distinguished on the basis of reactions with antisera, the pH stability of ATPase and succinatedehydrogenase activity. The gradual transition in histochemical characteristics between small and large pink fibres and the occurrence of stadia intermediate between myosatellite cells and small muscle fibres suggest that the small fibres are a growth stage.

Density of nerve terminations was highest on red fibres, which is in accordance with physiological data. White axial muscle fibres had the lowest termination density. Pink fibres of the m. hyohyoideus had significantly more terminations per millimeter fibre length than pink fibres of the axial muscles.

Vesicle densities per μ^2 endplate profile and per μ synaptic contact were similar in all types. Endplates on white fibres had narrower synaptic clefts and vesicles that were slightly more elliptical than those on other types. The presence of swollen mitochondria in endplates on red head muscle fibres may reflect the continuous rhytmic activity of these fibres. The absence of subjunctional folds on slow as well as on fast fibres suggest that endplates on both types release but little acetylcholine per stimulus.

INTRODUCTION

Muscles of fishes, like those of other vertebrates, contain fast and slow fibres which have different myosin light chains (FOCANT *et al.*, 1976). Slow as well as fast fibres can be subdivided in types differing in histochemical, functional and biochemical aspects (MOSSE & HUDSON, 1977; KORNELIUSSEN *et al.*, 1978; AKSTER & OSSE, 1978; VAN RAAMSDONK *et al.*, 1980, 1982a and 1982b). Even within the same muscle different fibre types may be active in different movements (AKSTER & OSSE, 1978).

Muscles of the carp (*Cyprinus carpio* L.) have been the subject of much investigation (BALLINTIJN *et al.*, 1972; HAMOIR *et al.*, 1981; SIBBING, 1982; GRANZIER *et al.*, 1983), but scarce attention has been paid to the presence of a variety of fibre types in these muscles. Com-

parison of these biochemical, functional and physiological data with (immune-)histochemical information about the distribution of the different fibre types over these muscles will contribute to our understanding of the functional diversity of fish muscle.

As histochemical properties, especially the pH resistence of ATPase activity, of similar fibre types from different fishes may show some variance, determination of these properties is necessary for this comparison. In the present study the histochemical characteristics of carp muscle fibres were studied in the axial muscles, of which biochemical and electromyographical data are known (JOHNSTON *et al.*, 1977) and in parts of a head muscle, the m. hyohyoideus, of which the contraction velocities have been described (GRANZIER *et al.*, 1983).

Muscles of advanced teleosts are multiterminally as well as multiaxonally innervated (BONE, 1978). As the distribution of nerve endings along a muscle fibre greatly influences the physiological properties, the innervation density of the different fibre types was determined by means of a reaction for acetylcholinesterase activity. The ultrastructure of endplates on different fibre types is also described.

MATERIAL AND METHODS

Common carp (*Cyprinus carpio* L.) of 15-27 cm standard length, bred in the laboratory and kept in tanks at 20°C, were used.

Muscles

Pieces of axial muscle containing red, pink and white fibres were taken from the tail region near the anus, as indicated in figs 1a and 1b. The studied parts of the m. hyohyoideus are shown in fig. 1c; for a description of this muscle see GRANZIER *et al.* (1983).

Experimental Procedure

Histochemistry

After anaesthetizing the fish with MS222, the muscles were rapidly removed. For histochemistry other than the reaction for acetylcholinesterase, pieces of the m. hyohyoideus were frozen and sectioned together with a piece of axial muscle (see figs 2b, 3).

Immuno-histochemistry was carried out on 6 μ-thick sections of muscle, fixed by freeze substitution, as described by POOL *et al.* (1980). This involves the indirect antibody method of STERNBERGER (1979) in combination with the histochemical reaction for peroxidase described by NAKANE & PIERCE (1966). Rabbit antisera against anti-red and anti-white carp myosin and against chicken pectoralis myosin were kindly provided by Dr. Van Raamsdonk (Department of Zoology, University of Amsterdam). These sera will subsequently be called anti-red, anti-white and anti-Pec. serum. The specificity of these sera has been described by VAN RAAMSDONK *et al.* (1980). The antisera were applied in a dilution of 1:10000 (anti-red), 1:500 (anti-white), and 1:800 (anti-Pec.) to muscle from 6 specimens.

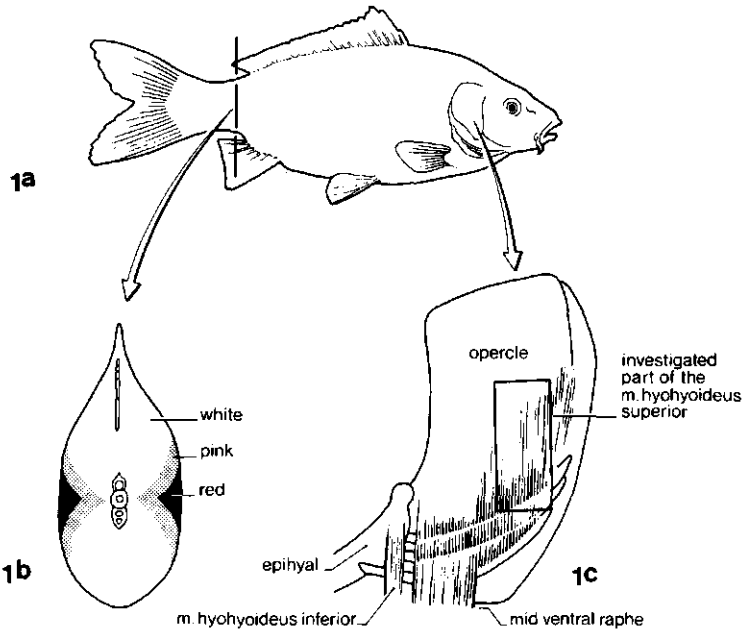


Fig. 1. Investigated muscle parts. *a.* the carp. *b.* position of the red (dark), pink (dotted) and white zones of the tail muscles. *c.* medial view of the opercular bones with the m. hyohyoideus.

Reactions for ATPase and for succinatedehydrogenase (SDH) were done on 10 μ sections of muscle parts from 7 specimens (frozen in freon, cooled in liquid nitrogen, and cut at -30°C).

The reaction for ATPase activity, modified after PADYKULA & HERMAN (1955) and GUTH & SAMAHA (1970), was carried out at 20°C. Sections of axial muscle combined with m. hyohyoideus superior were preincubated for 2 min. at pH's ranging from 4.2 to 10.6. As preincubation at the pH's 4.4, 10.3 and 10.4 proved useful in distinquishing fibre types, these pH's were used for further investigation. Succinatedehydrogenase (SDH) activity was determined according to the method of NACHLAS *et al.* (1957).

To demonstrate glycogen, the Periodic-acid-Schiff (PAS) reaction was applied to 6 μ sections cut adjacent to the sections used for immuno-histochemistry. There was no staining in control sections that had been preincubated with 0.5% α-amylase prior to the PAS reaction. Acetylcholinesterase activity was determined according to KARNOVSKI (1964) on muscle parts fixed for 1 h. in KARNOVSKI's (1965) fixative as described below. With this method endplates as well as terminal parts of axons appeared to stain. Modifying the iso-ompa (tetra-isopropylpyrophosphoramide, an inhibitor of unspecific cholinesterases) concentration of the preincubation medium over a range of 10^{-6} M to 10^{-4} M did not change this pattern. Varying the incubation time or the substrate concentration resulted in changes in the staining intensity but caused no change in the staining pattern.

Determination of the number of nerve terminations

On all fibres the reaction for acetylcholinesterase revealed several area's with ramifications of a preterminal axonbranch. Each such area was counted as one termination (as done by ALTRINGHAM & JOHNSTON, 1981). The number of terminations was counted in teased fibre preparations of 10 fishes of 15-18 cm standard length. Preparations were made of the red, pink and white zones of the axial muscles (fibre length before fixation about 3-5 mm), of the pink zone of the m. hyohyoideus superior (fibre length about 8 mm) and of the red zone of the m. hyohyoideus inferior (fibre length about 12 mm). Longitudinal 30 μ-thick sections of these muscle parts were used to compare the innervation density on the middle and the end parts of muscle fibres. Terminations on the red fibres of the axial muscle were too poorly visible in these sections to be counted. In the other muscle parts no such differences were found. The number of terminations present along the fibre's length within a frame of 1.03 mm was counted in teased fibre preparations directly from the slide with a microscope magnification of 100 x . As the distance between terminations depends on the degree of muscle concentration, the sarcomere lengths were measured and all values were corrected to a sarcomere length (after fixation) of 2.2 μ.

Electronmicroscopy

The opercular bones with the m. hyohyoideus of 4 fishes were, slightly stretched, pinned on a layer of paraplast and immersed in KARNOVSKI's (1965) fixative. The same fixative was injected into the axial muscles directly caudad to the anus. After fixation at room temperature for about one hour, the dorso-caudal part of the m. hyohyoideus superior, the m. hyohyoideus inferior, bundles of white axial muscle fibres and pieces of axial muscle containing both red and pink fibres were dissected. Fixation was continued for 12-19 h. at 4°C in fresh fixative. After rinsing in cacodylatebuffer, postfixation in osmiumtetroxide and dehydration, the tissue was embedded in an epon mixture. Ultrathin sections were cut with a diamond knife on a Reichert OMU II ultramicrotome, stained with uranylacetate and leadcitrate and scanned for endplates on a Philips 400 transmission electronmicroscope (Electronmicroscopy unit of the Institute of Agricultural Engineering, Wageningen). Shrinkage, estimated by comparing the measured actin filament length with the number of 40 nm periods present on this filament, was about 20%. Synaptic vesicles were traced, over the membrane, with a magnification of about 300000. Maximum and minimum diameter of these tracings were measured and used to calculate vesicle surface area and a mean diameter. The width of the synaptic cleft was measured opposite the presynaptic densities upon which synaptic vesicles in the endplates converge (HEUSER & REESE, 1973; PFENNINGER, 1973). The surface of the endplate profiles and the length of the synaptic contact were measured with a Summagraphics Supergrid datatablet in combination with a Minc 11 computer (Digital Equipment).

The significance of the measured differences was determined with Student's T test or with an approximate T test for unequal variances (SOKAL & ROHLF, 1969).

RESULTS

Fibre Type Characterization

(1) *Reaction with antisera.* All muscle fibres reacted intensely with either the anti-red or the anti-white serum. No fibres that reacted appreciably with both these antisera were found. The red zone of the axial muscles, the central part of the m. hyohyoideus inferior and a

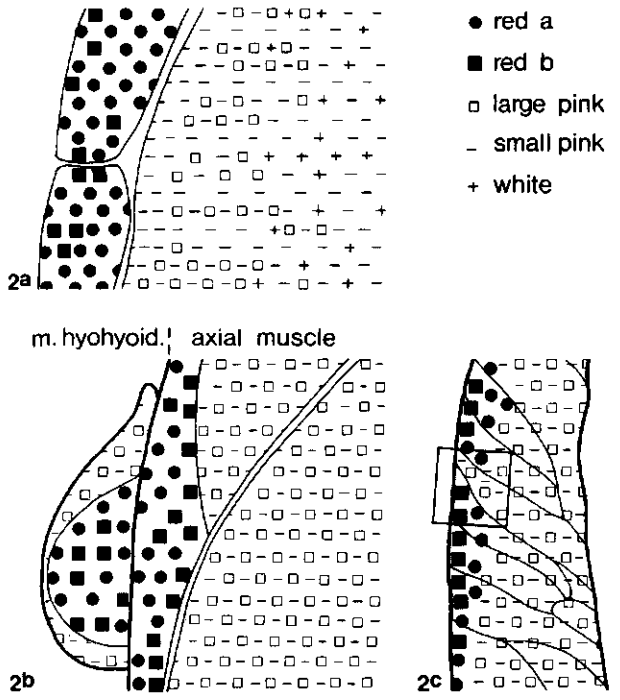
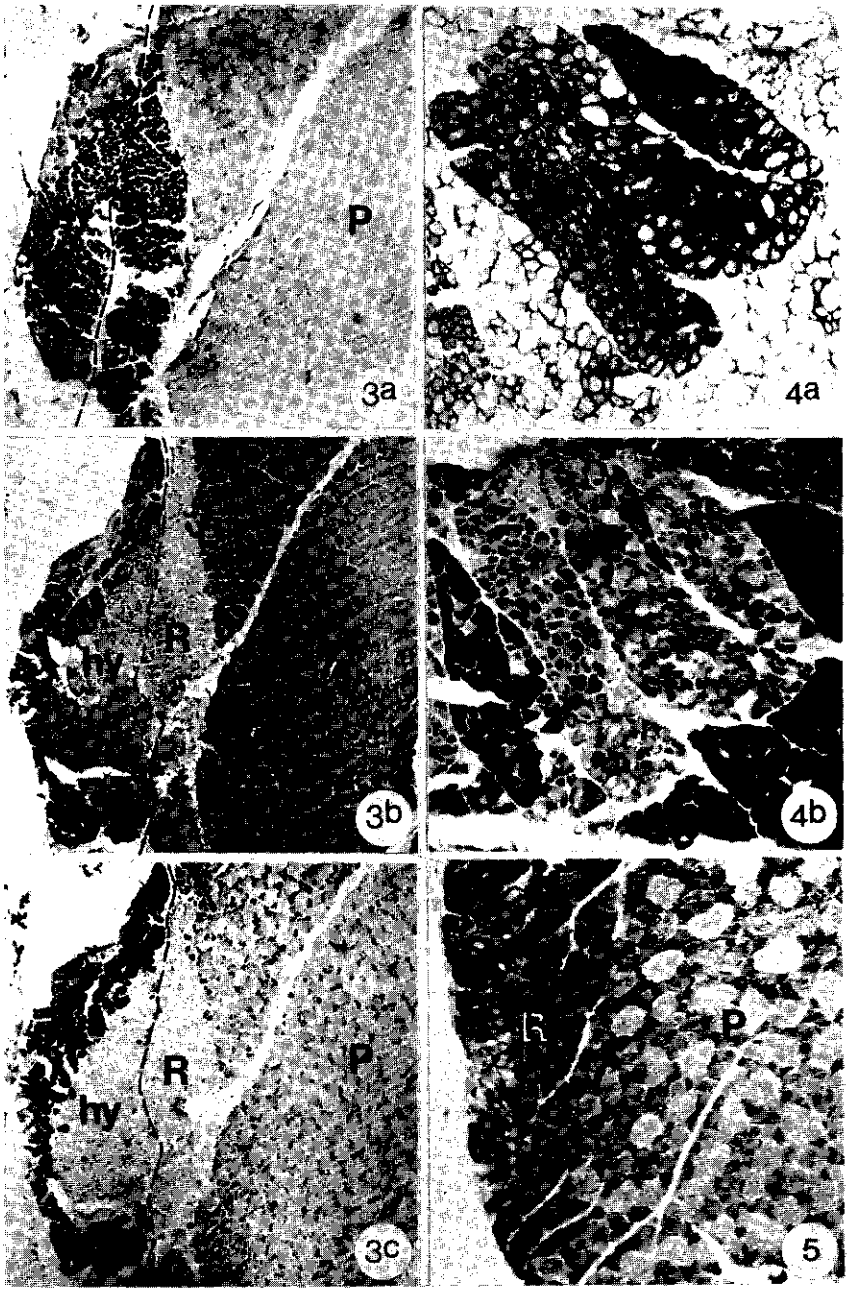


Fig. 2. Schematic representation of the fibre type composition of: *a.* the axial muscles (area corresponding to fig. 6), *b.* the m. hyohyoideus inferior combined with axial muscle (area corresponding to fig. 3) and *c.* the dorsocaudal part of the m. hyohyoideus superior (the rectangle corresponds to figs 8 and 9).

Fig. 3. From left to right, immuno-histochemistry of: the m. hyohyoideus inferior (hy) and the red (R) and pink (P) zones of the axial muscles (area corresponding to fig. 2b). *a.* anti-red serum, *b.* anti-white serum, *c.* anti-Pec. serum. Fibres react either with the anti-red or with the anti-white serum; the white zones of the m. hyohyoideus stain more intensely with the anti-Pec. serum than fibres of the axial muscles. Of the latter, only the small pink fibres stain. 20 ×.

Fig. 4. M. hyohyoideus inferior. *a.* SDH activity. *b.* ATPase activity after preincubation at pH 10.3. The red zone has a high SDH activity, it contains two fibre types differing in the pH resistence of their ATPase. The pink zone has a low SDH activity and an ATPase activity resistent to pH 10.3. 50 ×.

Fig. 5. Lateral axial muscle, red (R) and pink (P) zones. PAS reaction, red fibres contain more glycogen than pink fibres; small pink fibres more than large pink fibres. 150 ×.


P
3a
4a
R
hy
3b
4b
R
P
hy
3c
R
P
5

small medial zone of the m. hyohyoideus superior reacted with the anti-red serum. The other muscle parts reacted with the anti-white serum (figs 3a, 3b, 8a and 8b).

The anti-Pec. serum reacted more intensely with the m. hyohyoideus than with the axial muscles (fig. 3c). In the latter it generally reacted with the smallest fibres of the pink and the white zones, but in several specimens, groups of larger fibres of the pink zone also reacted with this antiserum. Fibres of the m. hyohyoideus, which reacted with the anti-white serum, also reacted with with the anti-Pec serum; large fibres reacted rather weakly, reaction intensity increased with decreasing fibre size. Some very small red-type fibres on the medial rim of the m. hyohyoideus superior also reacted with the anti-Pec serum (figs 8a, 8b and 8c).

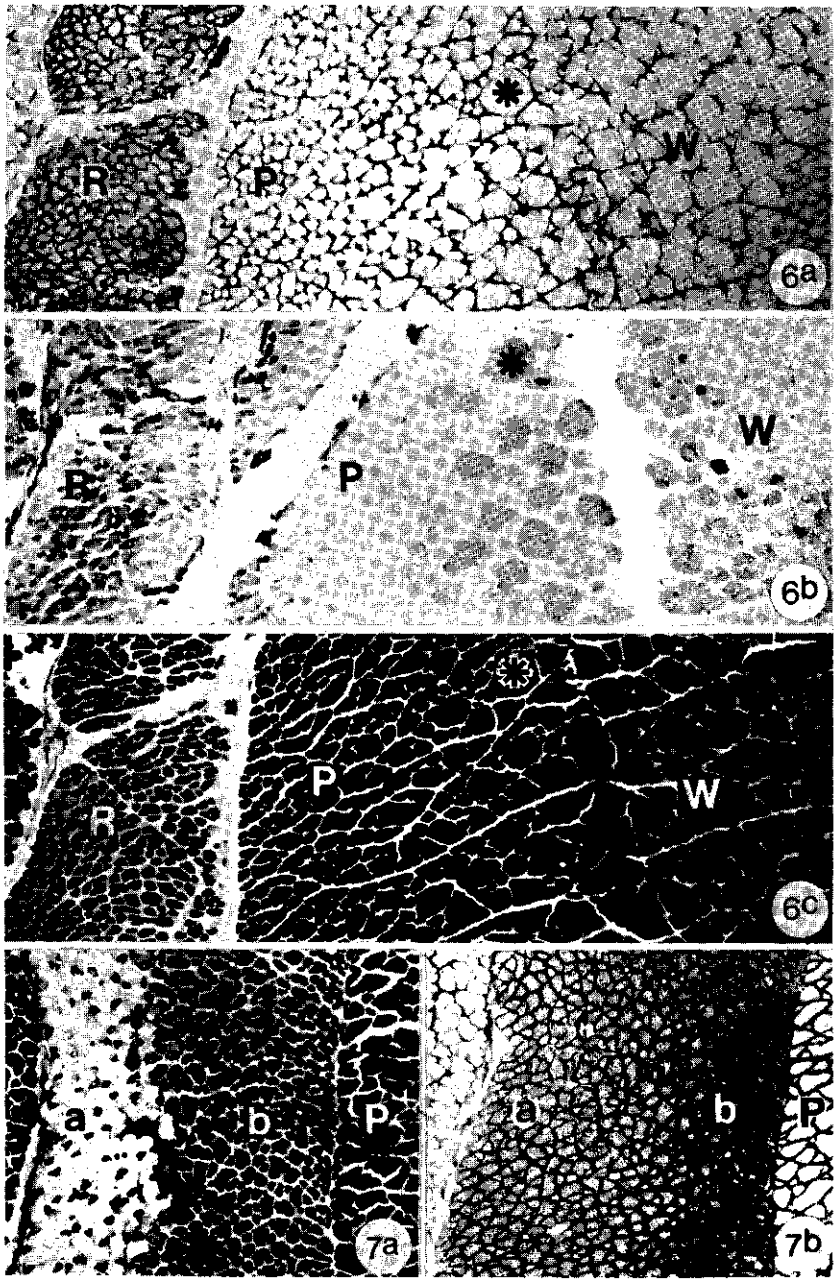
(2) *Myosin ATPase activity.* After a reaction for ATPase with preincubation at the same pH as that of the incubation medium, some fibres of the red zones stained less darkly than other fibres (fig. 6c).

(3) *Acid preincubation.* After preincubation at pH 4.4, large fibres of the pink zone of the axial muscles were more inactivated than other fibres (fig. 6b). The same applies to large fibres of the m. hyohyoideus which react with the anti-white serum (fig. 9b). It can now be concluded that they are pink fibres. After preincubation at pH 4.2 all fibres were inactivated.

(4) *Alkaline preincubation.* After preincubation at pH 10.3, white and pink fibres retained their activity. Some fibres of the red zones were inactivated, but other red fibres retained a great deal of their activity (figs 4b, 7a and 9c); they became completely inactivated at pH 10.4. At this pH the large fibres from the pink zones also lost part of their activity. At pH 10.6 all fibres became inactivated.

Fig. 6. Lateral axial muscles: red (R), pink (P) and white (W) zone (area corresponding to that in fig. 2a). *a.* SDH activity; the red zone has a high activity, the white zone has a low activity and in the pink zone a gradient is seen. *b, c.* ATPase activity. *b.* preincubation at pH 4.4; large fibres of the pink zone stain lighter than other fibres. *c.* preincubation at pH 9.4. A few fibres of the red zone show less ATPase activity than other fibres. 40 ×. Asterisks indicate the same fibres in the section series.

Fig. 7. Red (a, b) and pink (P) zones of the axial muscles. *a.* ATPase activity after preincubation at pH 10.3. Pink fibres retain their activity, the red zone contains two types (a and b) differing in activity. *b.* SDH activity. Red type b fibres have a higher activity than red type a fibres. 60 ×.


R
P
W
6a
R
P
W
6b
R
P
W
6c
a
b
P
7a
a
b
P
7b

(5) *Succinate dehydrogenase activity*. Red zones had a high SDH activity. Red fibres with an ATPase activity resistent to pH 10.3 had a higher SDH activity and a smaller size than the other red fibres (figs 7a, 7b and 9a, 9c). In fibres of pink and white zones, the SDH activity was located close under the sarcolemma. Small fibres of these zones had a higher SDH activity than larger fibres. In the pink zone of the axial muscles, the fibre size increased from lateral to medial and the SDH activity decreased in the same direction. The SDH activity of the white zones of the axial muscles and the pink zones of the m. hyohyoideus was very low.

(6) *Glycogen*. Red fibres were rich in glycogen which was mainly deposited close under the sarcolemma. Pink and white fibres contained less glycogen which was more evenly distributed over the fibre. The pink zone of the axial muscles contained more glycogen than the white zone; small pink fibres of the axial muscles contained more than large pink fibres (fig. 5). In the pink zones of the m. hyohyoideus, no difference in in glycogen content between small and large fibres was found.

Fibre types

On the basis of the reactions described above, three fast (pink and white) and two slow (red) fibre types were distinguished. The red types are the extremes in a range of intermediates. The same applies to the pink types. The characteristics of the fibre types are summarized in table I. The distribution over the muscle parts is given in figs 2a, 2b and 2c.

Innervation

The orientation of the axonbranches was predominantly perpendicular to the direction of the muscle fibres. They ran over several

Fig. 8. M hyohyoideus superior, area corresponding to rectangle in fig. 2c; asterisks, and arrows indicate the same fibres in the section series. Reactions with *a.* anti-red serum. *b.* anti-white serum. *c.* anti-Pec. serum. Fibres react either with the anti-red or with the anti-white serum. Small fibres of the white-reacting zone react intensely with the anti-Pec. serum, larger fibres stain less darkly. A few red fibres of the medial rim of the muscle part (arrows) also react with the anti-Pec. serum. 100 ×.

Fig. 9. M. hyohyoideus superior, similar zone as in fig. 8. *a.* SDH activity. *b, c.* ATPase after preincubation at: *b.* pH 4.4; *c.* pH 10.3. The ATPase of large pink fibres (*) is not resistent to preincubation at pH 4.4, the resistence increases with decreasing fibre size. The medial red fibres (b) have a more alkali resistent ATPase activity and a higher SDH activity than red fibres adjacent to the pink fibres (a). 150 ×.

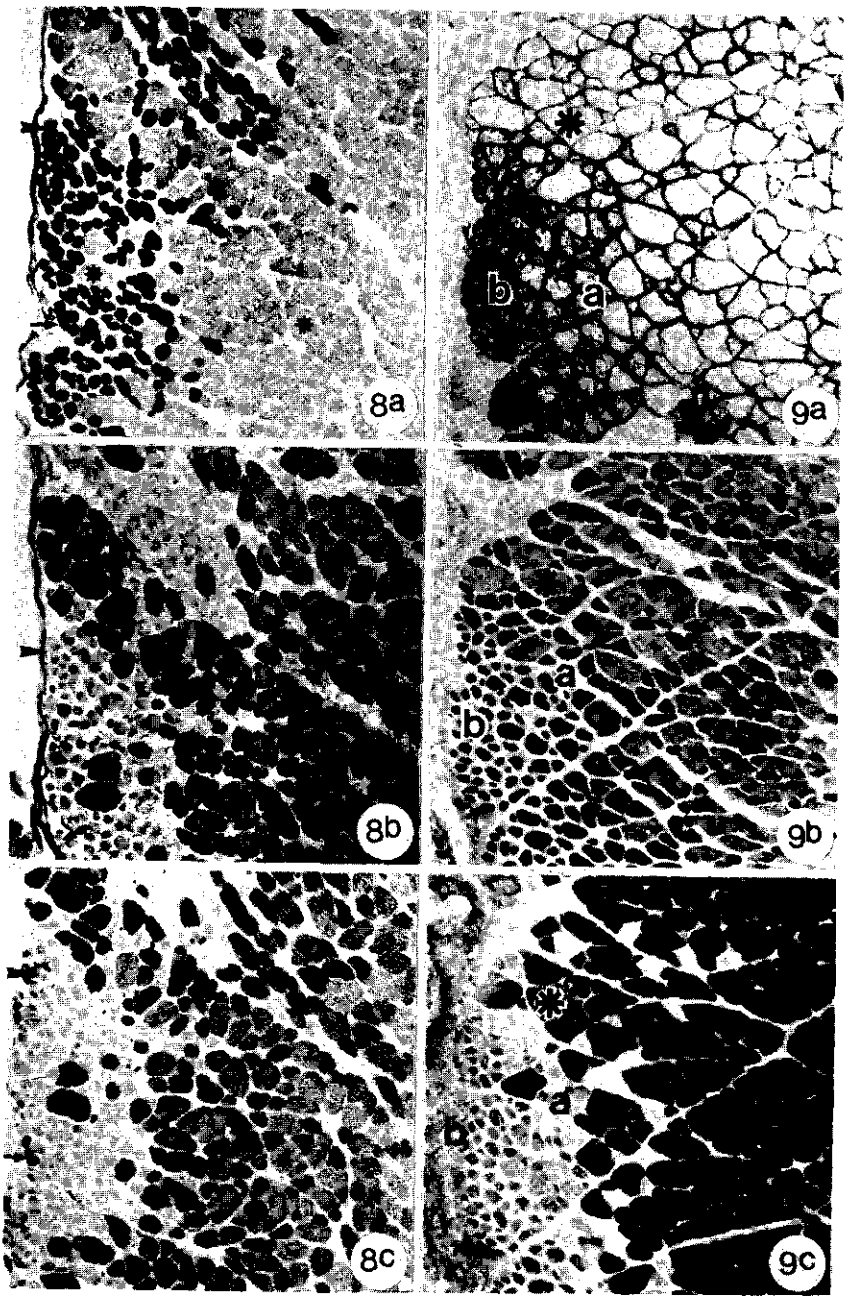

8a
b
a
9a
8b
a
b
9b
8c
a
9c

TABLE I

Histochemical characteristics of the fibre types.

		Diameter	*Reaction with antisera* anti-red	anti-white	anti-Pec.	*ATPase preincubation at pH* 4.4	9.4	10.4	*SDH*	*PAS reaction*
fast types:	white	+ + +	—	+ + +	—	+ +	+ + +	+ +	—	±
	large pink	+ + / + + +	—	+ + +	—/ + + [1]	±	+ + +	+	—/ ±	+
	small pink	± / +	—	+ + +	± / + + + [2]	+ / + +	+ + +	+ +	± / +	+ / + + [3]
slow types:	red a	+ +	+ + +	—	—	+	+ / + +	—	+ / + +	+ + +
	red b	+	+ + +	—	—[4]	+	+ + +	—	+ + +	+ + +

—, no or almost no reaction; ± → + + +, increasing reaction.

[1] large pink fibres of axial muscles mainly —/ ±, occasionally + / + +, large pink fibres of hyohyoid mainly + / + +.

[2] small pink fibres of the hyohyoid react more intensely than those of the axial muscles.

[3] small pink fibres of the axial muscles react more intensely than those of the hyohyoid.

[4] some very small red type b fibres of the rim of the hyohyoid superior react intensely with the anti-Pec. serum.

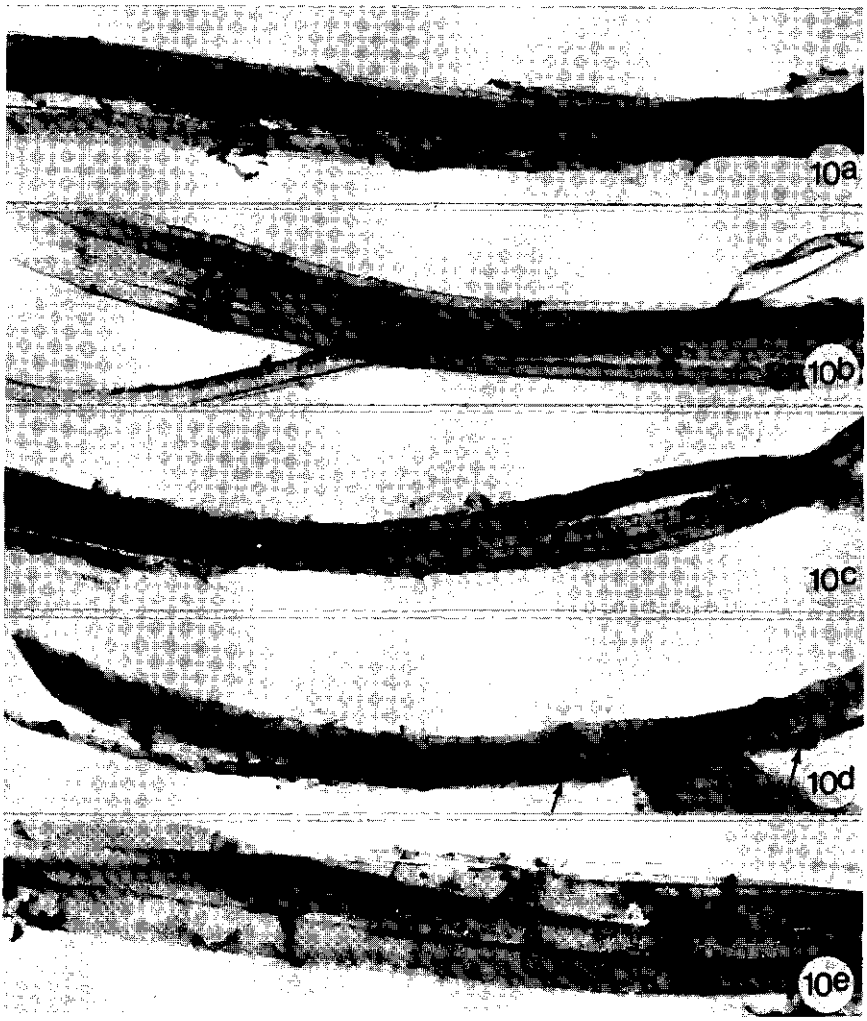


Fig. 10. Acetylcholinesterase activity, small bundless of teased fibres from: *a.* the red zone of the m. hyohyoideus inferior. *b.* the pink zone of the m. hyohyoideus superior. *c.* the red zone of the axial muscles. *d.* the pink zone of the axial muscles (note that a large and a small fibre are innervated by the same axonbranches, arrows). *e.* the white zone of the axial muscles. 100 × .

adjacent fibres of variable diameter and formed beadlike expansions on these fibres. On red fibres the direction of the axonbranches was slightly more longitudinal than on fibres of the other types (figs 10a-10e). The highest number of terminations (area's with ramifica-

tions of a preterminal axonbranch) per millimeter standardized fibre length was found on red fibres; pink fibres had fewer and the white fibres had the least. No significant difference was found between red fibres of the axial muscles and red fibres of the m. hyohyoideus. Pink fibres of the axial muscles had significantly fewer terminations per millimeter than pink fibres of the m. hyohyoideus (table II).

TABLE II
Number of nerve terminations per mm fibre length.

type	*n*	$\bar{x} \pm SD$	*average distance*
white axial	93	4.7 ± 1.4	213 μ
pink axial	74	5.0 ± 1.5	200 μ
pink hyohyoid	113	5.7 ± 1.5	175 μ
red axial	75	6.0 ± 1.9	167 μ
red hyohyoid	90	6.3 ± 1.5	159 μ

Significance red axial: red hyohyoid, NS; pink axial: pink hyohyoid, $P<0.001$; combined pink: white axial, $P<0.001$; white + pink: combined red, $P<0.01$.

Ultrastructure

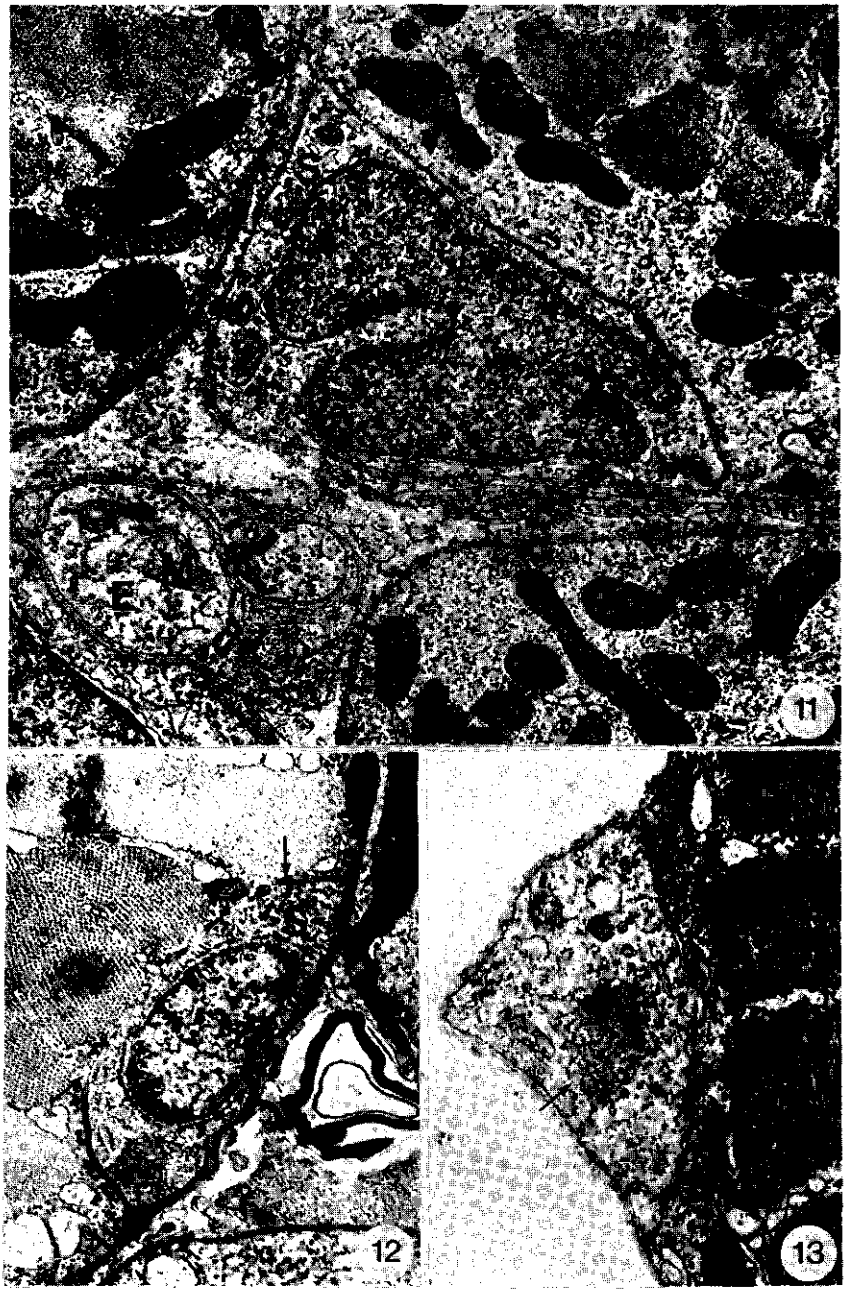
Red fibres could easily be distinguished from pink and white fibres by their greater number of mitochondria. Some red fibres contained fat droplets. In addition to accumulations of mitochondria, glycogen granula and polyribosomes were found close under the sarcolemma (fig. 11). In white and in pink fibres, most of the glycogen is found between the myofibrils (fig. 14) as is in agreement with the pattern obtained by histochemistry.

More capillaries, fibrocytes and collagen fibres were found between the red fibres than between the pink or the white fibres. Adjacent to fibres of all types and included within their basal lamina occurred

Fig. 11. Endplate (E) and myosatellite cell (S) on red fibres of the m. hyohyoideus superior. 18000 ×. g, glycogen; mi, mitochondria. Note the absence of basal lamina between the muscle fibre and the satellite cell.

Fig. 12. Myosatellite cell (S) on a white fibre of the m. hyohyoideus. The space between both cell membranes is partly occupied by basal lamina (arrows). 21000 ×.

Fig. 13. White fibre of the axial muscles with a satellite fibre containing myofilaments (arrow) which is included in the muscle fibres basal lamina. 21000 ×.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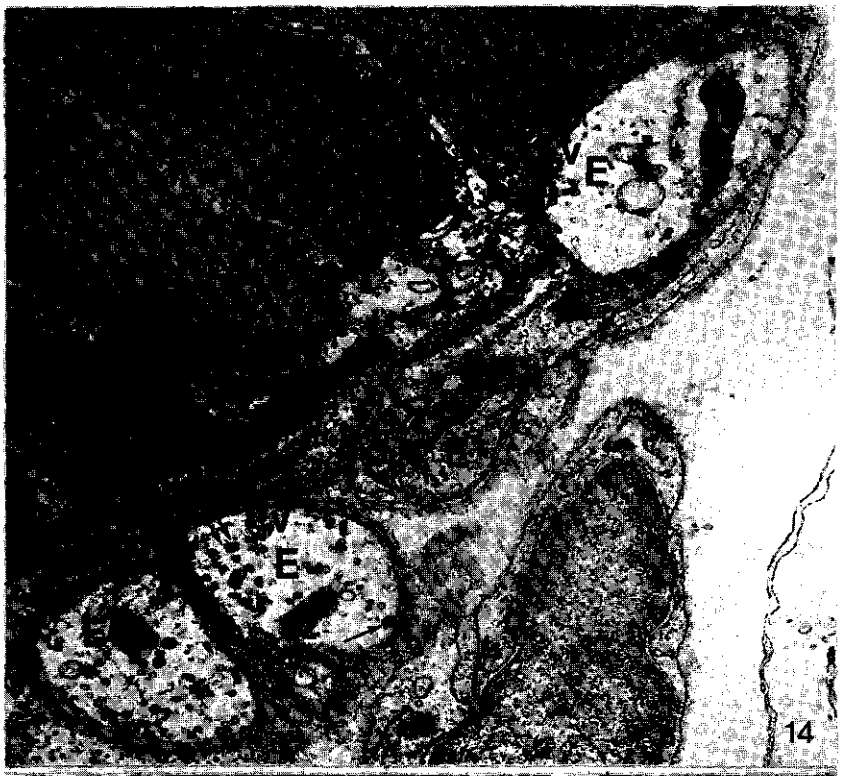
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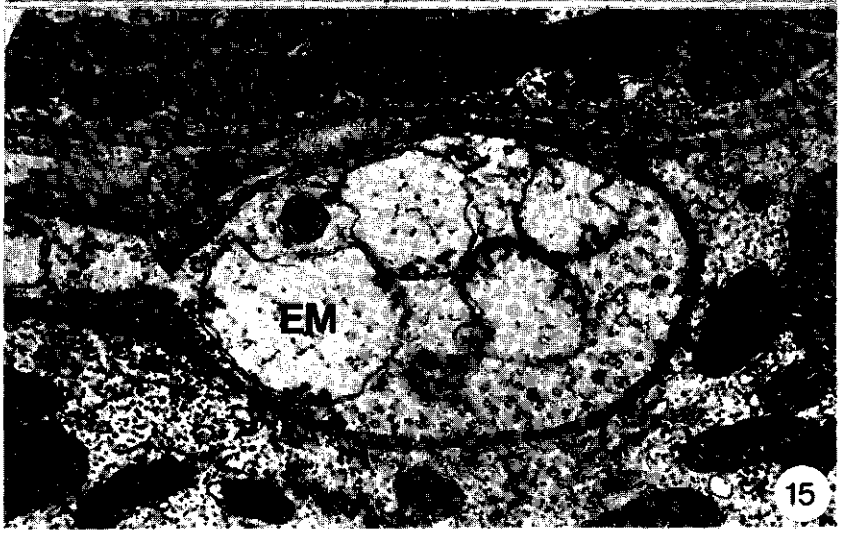
EM
15

myosatellite cells (fig. 11). They were seen with about the same frequency as endplates. Cells intermediate in structure between myosatellite cells and small muscle fibres were also noticed (figs 12 and 13).

Endplate profiles on slow (red) as well as on fast (white and pink) fibres were very variable in size (table III). They contained mainly clear vesicles with a diameter of about 50 nm. Vesicles in endplates on white fibres were slightly more elliptical than those in endplates on other types (table IV). Dense cored and clear vesicles with a diameter of about 80 nm. occurred (seldom) in endplates on all types (fig. 14). They were not included in the tables III and IV. In endplate profiles on red fibres more vesicles were found than in those on pink and white fibres; but in the number of vesicles per μ^2 endplate profile (corrected for mitochondria) or per μ synaptic contact no significant difference was found (table III). Endplates on red fibres of the m. hyohyoideus inferior often contained swollen mitochondria with disrupted cristae (fig. 15).

The width of the synaptic cleft, measured opposite the presynaptic densities upon which vesicles converge, was significantly smaller in white fibres (table III) than in pink or red fibres. Subjunctional folds were absent; only occasionally a shallow, bud-like invagination of the sarcolemma was seen. Inside the muscle, more dense material was attached to the sarcolemma beneath the endplate than elsewhere. The number of micropinocytotic vesicles was smaller beneath the endplate than in the rest of the sarcolemma.

DISCUSSION

Fibre Types

Of the three fast and two slow muscle fibre types distinguished in this study, white fibres were found only in the axial muscles. The other types occurred in both muscles. Small pink fibres were always found in

Fig. 14. Endplates (E) on white fibres of the axial muscles. v, translucent vesicles; arrow, dense cored vesicle; double arrow, large clear vesicles; g, glycogen, m, mitochondrion. Note the absence of sub-junctional folds. 21000 × .

Fig. 15. Endplate on a red fibre of the m. hyohyoideus inferior showing swollen mitochondriae with disrupted cristae. Note the difference between the endplate mitochondrion (EM), the muscle mitochondrion (MM) and the mitochondria in fig. 14.

TABLE III

Measurements on endplates, $\bar{x} \pm SD$. Data of hyoid and axial muscles combined.

type	*size of profile surface* μ^2	*n*	*vesicles per profile*	*n*	*vesicles per* μ^2 *profile*[1]	*n*	*length of synaptic contact* μ	*n*	*vesicles per* μ *contact*[2]	*n*	*width of synaptic cleft* nm	*n*
white	0.96 ± 0.71	24	51 ± 36	24	87 ± 70	24	1.70 ± 1.04	24	26 ± 20	24	60 ± 7	16
pink	1.26 ± 0.99	23	66 ± 45	23	74 ± 47	23	1.45 ± 0.63	23	32 ± 18	23	69 ± 12	16
red	1.53 ± 1.17	30	95 ± 30	30	77 ± 38	30	2.19 ± 1.66	30	26 ± 15	30	71 ± 12	22
white: pink[3]	NS		NS		NS		NS		NS		$P<0.05$	
white + pink: red[3]	NS		$P<0.01$		NS		NS		NS		$P<0.05$	

[1] corrected for mitochondria by subtracting mitochondria profile area.
[2] vesicles not separated from the presynaptic membrane by other celorganelles or "empty" spaces.
[3] statistic significance.

TABLE IV

Shape and size of synaptic vesicles, $\bar{x} \pm SD$. Data of hyohyoid and axial muscles combined.

	n		shape	size	
			ratio maximum/	calculated mean diameter nm.	
type	vesicles	(endplates)	minimum diameter	apparent	corrected[1]
white	668	(16)	1.26 ± 0.20	43.3 ± 11.2	57
pink	482	(12)	1.21 ± 0.17	44.0 ± 9.8	58
red	1516	(25)	1.22 ± 0.20	42.7 ± 12.1	56

Significance: white: pink, shape $P < 0.001$, size NS;
white + pink: red, shape $P < 0.01$, size $P < 0.05$.

[1] corrected for section thickness (about 8%, calculated after Jones and Kwanbunbumpen) and for shrinkage (about 20%).

combination with large pink or large white fibres. The same fibre types (except white) were found in other head muscles of the carp (AKSTER & SIBBING, 1982).

The classification of the fibres into types was mainly based on the immuno-histochemistry and the reactions for ATPase activity. In a former study on perch muscle (AKSTER & OSSE, 1978), the distribution of metabolic enzyme activity appeared to be not entirely in agreement with such a classification. In the present study of carp muscle, the difference in ATPase activity between both red types was accompanied by a difference in SDH activity in all investigated muscle parts.

That the pH stability of the ATPase of white fibres is greater than that of pink fibres is unexpected as in all other investigated fishes, pink fibres were found to have a more pH stable ATPase activity than white fibres (JOHNSTON *et al.*, 1974, 1975; MOSSE & HUDSON, 1977; KORNELIUSSEN *et al.*, 1978; AKSTER & OSSE, 1978; VAN RAAMSDONK *et al.*, 1980). However, distinctions between fibre types based on differences in the pH resistence of the ATPase activity are in agreement with those made on the basis of immuno-histochemistry (see also VAN RAAMSDONK *et al.*, 1980).

Red fibres

The type red b fibres are similar to the red fibres of *Cyprinus carpio* with a high ATPase activity described by WITTENBERGER & RUSU (1978). Perch muscle fibres with a high, pH resistent ATPase activity, as determined by histochemistry, were found to be active during slow movements (AKSTER & OSSE, 1978) and to have less T system/SR contact than the other red fibres (AKSTER, 1981). They are probably very slow fibres. As red type b fibres of the carp have less T system/SR

contact than fibres from a mixed (red a and red b) muscle part (AKSTER *in prep.*), it is very probable that also in the carp the red b fibres are slower than the red a fibres. The relatively high SDH activity and the high amounts of glycogen found in all red fibres are very probably related to the high resistance to fatigue of these fibres as compared with that of pink and white fibres (GRANZIER *et al.*, 1983). Red type b fibres, which have the highest SDH activity, can be expected to have the best endurance.

Pink and white fibres

The mosaic of small and large fibres, present in the pink and white zones of *Cyprinus carpio*, has also been described for other fishes. As the small fibres have often higher activities of metabolic enzymes or more glycogen than the larger fibres (PATTERSON *et al.*, 1975; MOSSE & HUDSON, 1977; KORNELIUSSEN *et al.*, 1978; EGGINTON & JOHNSTON, 1982) a functional diversity between small and large fibres was suggested (BODDEKE *et al.*, 1959; BONE *et al.*,1978). The innervation of small and large fibres by the same axonbranches (see fig. 10d) could argue against a difference in function, but innervation of these fibres by several, functionally different, neurons as described for amphibian muscle (RIDGE & THOMPSON, 1980a, b) can not be excluded. Another explanation for the presence of these small fibres, is that they may represent a growth stage. This is based on the increase in fibre number in growing fish (GREER-WALKER, 1970; WILLEMSE & VAN DEN BERG, 1978; WEATERLY & GILL, 1981), on the gradual transition of both fibre size and pH stability of the ATPase activity from small to large fibres (CARPENE & VEGGETTI, 1981; also found in the present study, fig. 9b) and on the greater fractional volume of nuclei in the small fibres (EGGINTON & JOHNSTON, 1982b). NAG & NURSALL (1972) and KRYVI & EIDE (1977) suggest that in fishes new muscle fibres arise from myosatellite cells. The presence of myosatellite cells and of transition stages between myosatellite cells and small muscle fibres in the studied muscles of *Cyprinus carpio* strongly supports the theory of the small fibres being a growth stage. The anti-Pec serum, which reacts more intensely with small than with large pink fibres, is known to react with the entire pink zone of *Brachidanio rerio* (VAN RAAMSDONK *et at.*, 1982) and also, transiently, with developing muscle fibres of *Barbus conchonius* (BARENDS *et al.*, 1983). The available data about the anti-Pec serum are as yet insufficient to justify conclusions about the function of the small fibres. It may be that they are developing fibres which have a different function from that of the more mature fibres.

The contraction velocity of nerve-muscle preparations from the "white" part of the m. hyohyoideus superior was about twice that of

similar preparations from the red zone of the m. hyohyoideus inferior (GRANZIER *et al.*, 1983). From the present study, it appears that "white" parts of the m. hyohyoideus have a greater resemblance to the pink zone of the axial muscles than to the white zone. As the pink axial muscles of the carp are intermediate between the red and the white muscles in function as well as in biochemically determined ATPase activity (JOHNSTON *et al.*, 1977) it can be expected that the white fibres of the axial muscles have a higher contraction velocity than the fast fibres described by GRANZIER *et al.* (1983).

Innervation

All studied fibre types of the carp appeared to be multiply innervated. The highest termination density was found on red fibres, pink fibres came next and white fibres had the lowest density. The pink fibres of the m. hyohyoideus had significantly more terminations per millimeter fibre length than the pink fibres of the axial muscles (table II). The data obtained in this study are similar to those reported by NAKAJIMA (1969) for red and white pectoral fin muscles of the snake fish *Ophiocephalus argus*, which have respectively 5.9 and 4.2 terminations per millimeter fibre length. For the, longer, fast axial muscle fibres of *Cottus scorpius* a larger distance between terminations (640 μ) was found (HUDSON, 1969).

There is electrophysiological evidence that the innervation of muscle fibres of advanced teleosts is polyneural (TAKEUCHI, 1959; HAGIWARA & TAKAHASHI, 1967; HUDSON, 1969). Investigation of fast axial muscle fibres of *Cottus scorpius*, which have 8 to 23 terminations per fibre, indicated that each fibre is innervated by 8 to 20 different axons (HUDSON, 1969). As the axial muscle fibres of the studied carp are about 3-5 mm long, there are about 10-20 terminations per fibre which is in the same range as that on the axial muscle fibres of *Cottus scorpius (*HUDSON, 1969) and *Gadus virens* (ALTRINGHAM & JOHNSTON, 1981). Fibres from the superior and inferior parts of the m. hyohyoideus are about 8 and 12 mm long, respectively. This means that they have about 35 and 60 terminations per fibre, respectively. In the present study it is not possible to discriminate between polyneural (several endplates per fibre, each of a different axon) and multiterminal (several endplates from the same axon) innervation. In the nerve which innervates the hyohyoideus (ramus hyoidei nervi facialis, VETTER, 1878), extensive branching of axons was noted (AKSTER & VAN DOMMELEN, *unpubl. results*). This suggests that the great number of terminations per fibre in the m. hyohyoideus is at least partly multiterminal innervation.

GRANZIER *et al.* (1983) found that both white (pink) and red muscle fibres of the m. hyohyoideus of the carp respond to a single stimulus with a twitch after neural as well as after epimuscular stimulation. With epimuscular stimulation, however, a supramaximal stimulus amplitude could not be found for pink or for red fibre bundles. The percentage of the maximum tension, found by stimulation via the nerve, that could be obtained by epimuscular stimulation was higher for pink fibre bundles than for red fibre bundles. This suggests that in pink fibres, depolarization of the sarcolemma is conducted over a greater distance than in red fibres. This would necessitate a higher density of nerve terminations on the red fibres, a prediction that is confirmed by the present study. It is probable that more localized activation of muscle fibres results in a finer regulation of the contraction of these fibres.

Ultrastructure of Endplates

The differences in ultrastructure between endplates on different fibre types of the carp are very small and can not be related to differences in physiological properties as well as those of birds and mammals. Terminals on focally innervated fast avian muscle fibres are described to be larger and to contain more vesicles than those on multiply innervated tonic fibres; they also release more acetylcholine per stimulus (VRBOVÁ *et al.*, 1978). The postjunctional membrane of the fast fibres, which is more sensitive to acetylcholine than that of the tonic fibres (VRBOVÁ *et al.*, 1978), is folded, whereas that of the tonic fibres is relatively smooth (HESS, 1970). Between fast and slow twitch fibres of mammals also structural differences are found. Endplate profiles on fast twitch fibres have larger sizes, more and deeper postjunctional folds and a greater abundance of synaptic vesicles than those on slow twitch fibres (PADYKULA & GAUTHIER, 1970). No such differences were found between fast (white and pink) and slow (red) muscles of the carp.

Absence of subjunctional folds, as found in all fibre types of the carp, was also described for multiply innervated fibres of other teleosts (GAINER & KLANCHER, 1965; NISHIHARA, 1967; NAKAJIMA, 1969; KORDYLEWSKI, 1974). The absence of subjunctional folds, which provide space for acetylcholine receptors and acetylcholinesterase, suggests that endplates on fast as well as on slow multiply innervated fish muscle fibres release but little acetylcholine per stimulus.

Red fibres of head muscles of fishes are active during respiration, a continuous rhytmic movement (AKSTER & OSSE, 1978; BARENDS, 1979). The swelling of mitochondria in these fibres may reflect a

greater sensitivity to distortion by the fixation medium of active than of relatively inactive mitochondria and so represent the continuous activity of the red head muscle fibres.

Vesicle size and shape are related to the type of neurotransmitter which they contain (PFENNINGER, 1973). Also, in insects, vesicles in slow excitatory endings are smaller than those in fast endings (TITMUS, 1981). As the differences between vesicles in endplates on different fibre types of the carp are very small, it is not possible to relate them to differences in activity pattern of the fibre types. Size and shape of the vesicles in the carp neuromuscular junctions are similar to those of vesicles in other vertebrate cholinergic junctions (JONES & KWANBUNBUMPEN, 1970; BEST & BONE, 1973; PFENNINGER, 1973).

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Contractile Properties of a White- and a Red-Fibre Type of the M. Hyohyoideus of the Carp (*Cyprinus carpio* L.)

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Summary. Isometric contraction parameters were measured for white and red fibre bundles isolated from the m. hyohyoideus of the carp. The two fibre types, which have multiterminal innervation, were stimulated via the nerve as well as epimuscularly. Both red and white fibres reacted to a single stimulus with a twitch. Stimulation via the nerve revealed:

1. Twitches and tetani of white fibres have shorter contraction and relaxation times than those of red fibres.

2. Both types reach similar maximal tetanic tensions (about 12 N/cm^2) but red fibres require a higher stimulus frequency to reach this tension.

3. The ratio of twitch tension to maximum tetanic tension is 0.42 for white and 0.27 for red fibres.

4. The maximum slope of tension rise in white fibres is independent of the stimulus frequency; in red fibres it increases at high stimulus frequencies.

5. White fibres are more susceptible to fatigue than red fibres. After about 45 s of repeated tetanization (22 tetani) white fibres had lost half their tension. Red fibres had lost half their tension after about 10 min (300 tetani).

6. Sag, the decline of tension during a tetanus, is greater in white than in red fibres. It has a different frequency dependence in both types.

7. Epimuscular stimulation resulted in a slow, incomplete contraction and a very slow decline of tension, especially in red fibres.

8. In agreement with existing biochemical, electromyographical and ultrastructural data, white fibres are adapted for quick short duration activity and red fibres for slow, sustained activity.

Introdution

In fishes, as in other vertebrates, red and white muscle fibres are found (Focant et al. 1976). In both types subtypes are distinguished on the basis of biochemical, histochemical and ultrastructural characteristics (Patterson et al. 1975; Bone 1978; Akster and Osse 1978; Barends 1979; van Raamsdonk et al. 1980; Akster 1981; Hamoir et al. 1981; Johnston 1981).

Studies aimed at understanding the relation between different types of movement like swimming at different velocities, feeding, breathing, and the type of muscle fibre employed in each of these movements have shown that generally white fibres are active during fast movements whereas red fibres are employed in relatively slow, repetitive or prolonged activities (Bone 1966; Johnston et al. 1977; Akster and Osse 1978). Multiterminally innervated white fibres, however, are found to be already active at sustainable swimming speeds (Hudson 1973; Bone et al. 1978; Johnston and Moon 1980a, b).

Studies concerning the mechanical properties of different fibre types of fish muscle are scarce. This is mainly due to experimental difficulties. In other vertebrates limb muscles offer the advantages of being easily approached, having a clearly defined fibre orientation and an accessible and relatively simple nerve supply. Fish muscle, however, has a complex and as yet partly unknown innervation (Bone 1978). Moreover, myotomal muscles have a complex fibre orientation and myoseptal insertion.

The present study was undertaken to fill, at least partly, this gap in our knowledge. For our experiments we selected a head muscle, the m. hyo-

hyoideus, which is situated ventrally at the inner side of the opercular (Fig. 1 A, B). This muscle has a parallel fibre orientation, attaches at both ends to bony elements and is innervated by a long, easily accessible nerve. Histochemical fibre typing, following the experiments, showed that almost pure white or red bundles could be obtained from this muscle. Stimulation of these fibres by means of the nerve gave reproducible twitches and tetani, which can be compared with biochemical and ultrastructural data. In this way a better understanding of the relation between the fibre types present in fish muscle and the functional demands imposed upon these muscles can be obtained.

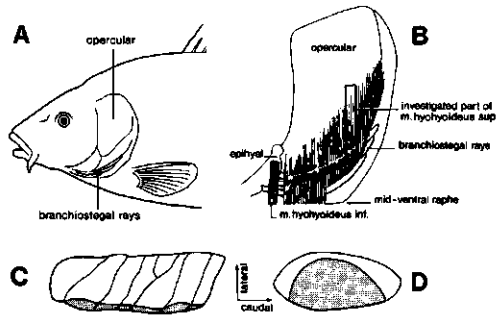

Fig. 1 A–D. Position and composition of the m. hyohyoideus. **A** Opercular in situ. **B** Medial view of the opercular bones with the m. hyohyoideus. **C, D** Fibre type distribution in the dorso-caudal part of the m. hyohyoideus superior (**C**) and in the m. hyohyoideus inferior (**D**): *dotted region:* red fibres; *clear region:* white fibres

Materials and Methods

Muscle Structure. The m. hyohyoideus is divided in a superior and an inferior part (Fig. 1 B). The superior part is subdivided in three parallel-fibered sheets of muscle connecting the opercular and the subopercular with the three branchiostegal rays. The rostral part of the third sheet inserts finally at the mid-ventral raphe. In carp of about 20 cm standard length the dorso-caudal part of this muscle contains mainly white fibres, only a narrow superficial zone of red fibres is present (Fig. 1 C). The more rostrally situated m. hyohyoideus inferior originates from the inner side of the epihyal and inserts likewise on the ventral raphe. It consists of a bundle of red fibres, surrounded by white fibres at its ventral, lateral and caudal sides (Fig. 1 D). The muscle fibres of both parts are multiterminally innervated (Akster, in preparation) by a long branch of the facial nerve (ramus hyoidei nervi facialis; Vetter 1878). This nerve can easily be dissected from the anterior edge of the opercular. White fibres of the m. hyohyoideus resemble the superficial white, also called pink, fibres of the myotomal muscles in the histochemical characteristics of their myofibrillar ATPase activity, but not in their succinate dehydrogenase activity. The m. hyohyoideus contains two types of red fibres which differ in histochemical staining for ATPase (Akster, in preparation). Both muscle parts are active in the contraction phase of the respiratory cycle during increased ventilation (Ballintijn 1969a, b).

Experimental Procedure. Carp (*Cyprinus carpio* L.) measuring 17 to 25 cm were kept in tanks at 20 °C for at least one month. The fishes were quickly anaesthetized with 0.1% MS222 (Sandoz) and the opercular bones were rapidly dissected. White fibre bundles were prepared from the upper caudal part of the m. hyohyoideus superior by carefully transecting the origo of the superficial layer of red fibres and of the white fibres adjacent to the studied bundle and also by removing the upper branchiostegal ray on both sides of the insertion of this bundle (Fig. 2). In this way the innervation of the investigated fibre bundle was retained and the mechanical responses of only these fibres were measured. Similarly red bundles of the m. hyohyoideus inferior were separated from the surrounding white fibres by carefully transecting the insertion of the latter. In this way bundles consisting of approximately 94% white or red fibres were obtained. This was measured by histochemical staining for succinate dehydrogenase activity (Nachlas et al. 1957) after each experiment.

The fibre bundles were kept at 20 °C in frequently refreshed saline containing 119 mM NaCl, 2.7 mM KCl and 4.0 mM $CaCl_2$, according to the estimated value of carp electrolyte composition (Houston et al. 1970). Also 15 mM glucose was added. The saline was oxygenated with a mixture of 95% O_2 and 5% CO_2 and then adjusted to pH 7.4 by adding $NaHCO_3$.

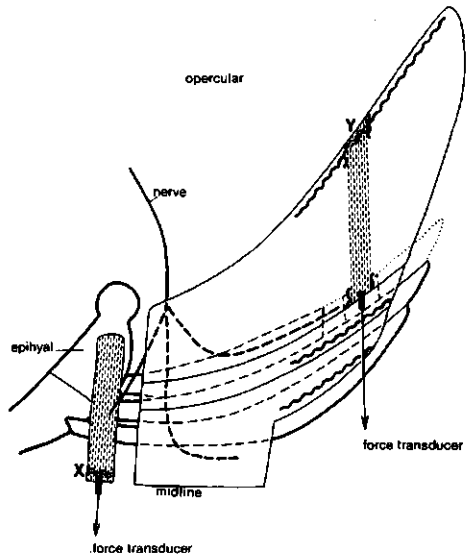

Fig. 2. Medial view of the nerve-muscle preparation: *hatched area:* investigated fibre bundles; ≈ ≈ X: area where the white fibres of the m. hyohyoideus inferior were sectioned; ≈ ≈ Y: area were the medial red fibres of the m. hyohyoideus superior were sectioned; ~~: areas where white fibres adjacent to the studied white fibre bundle of the m. hyohyoideus superior were sectioned. Parts of the upper branchiostegal ray adjacent to the insertion of the studied bundle were removed

Supramaximal stimuli were applied via the nerve to 14 white fibre bundles with a cross section area of 0.43 ± 0.16 mm^2 (mean $\pm$ SD) contaminated with 6.1 ± 2.8% (of this area) of red fibres, and to 12 bundles of red fibres with a cross section

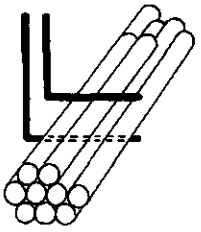

Fig. 3. Position of the electrodes in epimuscular stimulation

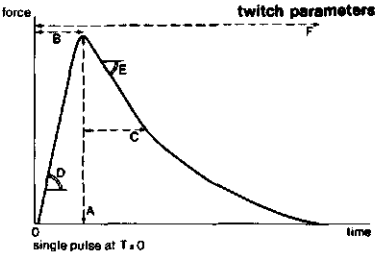

Fig. 4. Measured twitch characteristics, see Tables 1 and 3

area of 0.51 ± 0.21 mm^2 containing $5.6 \pm 2.8\%$ of white fibres. Isometric measurements were performed at the fibre length giving optimal twitch tension (L_0). For white bundles this was 7.6 ± 0.8 mm and for red bundles 12.8 ± 2.3 mm.

Epimuscular stimulation was performed with platinum electrodes (Ø 0.2 mm) as indicated in Fig. 3, after adding 10^{-4} M D-tubocurarine chloride to the saline. Twelve preparations of white fibres (cross section area 0.38 ± 0.18 mm^2; area occupied by red fibres $5.0 \pm 3.8\%$; $L_0 = 7.0 \pm 3.8$ mm) and ten bundles of red fibres (cross section area 0.55 ± 0.23 mm^2; area occupied by white fibres $5.6 \pm 3.3\%$; $L_0 = 13.0 \pm 2.6$ mm) were used.

The fibres were connected with tiny hooks, inserted in their tendons or in the remaining part of the upper branchiostegal ray, to a Grass force transducer Ft 03C. In experiments with stimulation by means of the nerve the force measurement was accurate to 0.1 mN and the transducer compliance was 0.5 mm/N. For epimuscular stimulation these values were 0.02 mN and 2.0 mm/N, respectively. Single stimuli or stimulus trains were applied at intervals of about 5 min. Contractile responses were recorded simultaneously with an UV recorder (Oscillograph SE 3006/DL) and a heat sensitive paper recorder (Watanabe WTR/711).

Identical twitch characteristics could be obtained over periods of 4–8 h. Results obtained after decline of the twitch tension were discarded. Fatigue sensitivity was studied with repeated tetanisation. Pulse trains of 40 Hz, lasting 0.5 s, were repeated every 2 s. During these experiments the saline was not refreshed to avoid interference with the recordings.

Results

Stimulation by Means of the Nerve

Twitches. Both fibre types reacted to a single stimulus with a twitch. White fibres were faster than red fibres, both rise and decline of tension occurred more rapidly (Table 1, Fig. 6). Administration of 10^{-5} M D-tubocurarine chloride blocked the nerve-muscle transmission within a few minutes; 10^{-4} M blocked almost immediately.

Pulse Train Responses. Maximal tetanic tension was similar for both types (Table 2). In white fibres it was reached at stimulus frequencies of 100–150 HZ and in red fibres at 200–300 Hz (Figs. 6D, 6E and 7). In white fibres the maximal positive slope of tension rise was similar in a twitch and in tetani of different frequency. In red fibres this slope increased with increasing stimulus frequencies higher than 100 Hz. At the frequency where maximum tetanic tension was reached this slope was 1.33 times that of a twitch.

Table 1. Stimulation by means of the nerve. Twitch characteristics (see Fig. 4), means ± SD. white, $n = 14$; red, $n = 12$

		White	Red
A. Maximum twitch tension	N/cm^2	5 ± 0.7	3 ± 0.7
B. Time-to-peak-tension	ms	32 ± 3	59 ± 8
C. Half relaxation time	ms	37 ± 5	106 ± 28
D. Maximum positive slope	N/cm^2 s	285 ± 53	103 ± 26
Relative positive slope (D/A)	s^{-1}	58 ± 8	34 ± 6
E. Maximum negative slope	N/cm^2 s	92 ± 29	19 ± 4
F. Twitch time	ms	176 ± 22	598 ± 104

Table 2. Stimulation by means of the nerve. Characteristics of maximal tetani (see Fig. 5). Means ± SD. white, $n = 14$; red, $n = 12$

		White	Red
G. Maximal tetanic tension	N/cm^2	12 ± 2.5	11 ± 2.5
H. Time-to-peak-tension	ms	178 ± 41	250 ± 47
I. Maximal positive slope	N/cm^2 s	304 ± 62	132 ± 32
I/D (see Table 1)	–	1.04 ± 0.07	1.33 ± 0.16
Maximal negative slope	N/cm^2 s	128 ± 31	65 ± 18
Ratio of twitch tension to tetanic tension	–	0.42 ± 0.07	0.27 ± 0.02

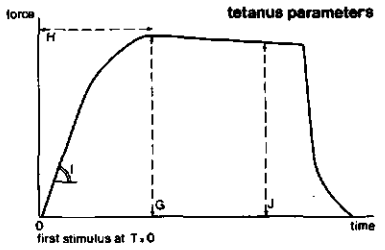

Fig. 5. Measured characteristics of tetani, see Table 2 and, for *J*, Fig. 7

In both fibre types a decline in tension during a tetanus was found; the tension reached an initial maximum and then declined or sagged to a lower level. This decline is frequency dependent and it was more marked in white than in red fibres. Sag of white fibres was maximal at 40 Hz; it decreased at higher frequencies. Sag of red fibres decreased from 15–50 Hz and then increased with increasing frequency (Fig. 7). Superimposing a 75 Hz tetanus upon a 40 Hz tetanus of white fibres just after the onset of sag resulted in an increase of tension to a level similar to that of a 75 Hz tetanus alone. In both fibre types tension of prolonged 40 Hz tetani which had sagged by about 80% of their initial value could be raised by increasing the stimulus frequency.

Resistance to Fatigue. Red fibres had a better resistance to fatigue than white fibres (Fig. 8). A fatigue index, as used by Burke et al. (1973), consisting of the ratio of the 120th and the first tetanic tension was 0.61 ± 0.08 for red and 0.12 ± 0.66 for white fibres.

Tetani of partly fatigued fibres of both types showed a decrease of the maximum rate of tension development. In red fibres also a striking increase of the time-to-peak-tension was found. In contrast to sagging tetani, tetani of fatigued fibres showed no increase in tension when the stimulus frequency was raised. Restoration of the original twitch and tetanus characteristics in frequently refreshed saline after a fatigue sensitivity experiment took about half an hour. After recovery a similar fatigue sensitivity test showed a 25% more rapid drop in tension.

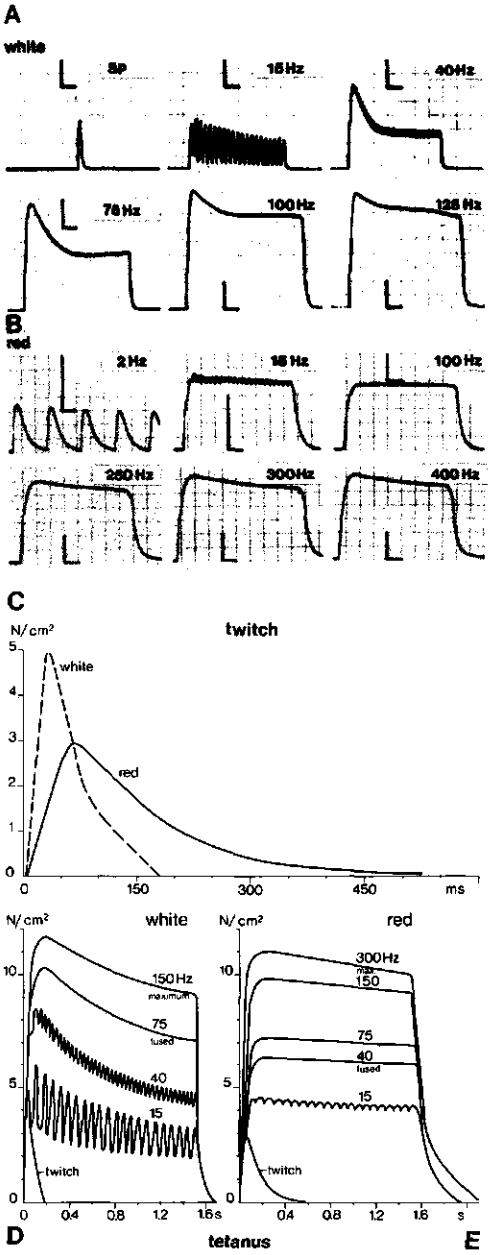

Fig. 6A–E. Responses to nerve stimulation. **A**, **B** Recordings of a white- (**A**) and a red (**B**)-fibre preparation; *vertical line:* 4 N/cm², *horizontal line:* 200 ms, note the scale differences! Both rise and decline of tension are faster in white than in red fibres. Tetani of white fibres show more sag than tetani of red fibres especially at low stimulus frequencies. **C**, **D**, **E** Schematic representation of twitches (**C**) and pulse train responses of white (**D**) and red (**E**) fibre bundles. White fibres have a higher twitch tension than red fibres. Fusion frequency is 60–75 Hz for white fibres and 40–50 Hz for red fibres. Maximal tetanic tension is reached at 100–150 Hz for white fibres and at 200–300 Hz for red fibres. In white fibres the maximal slope of tension rise is independent of the stimulus frequency. In red fibres this slope increases at high stimulus frequencies

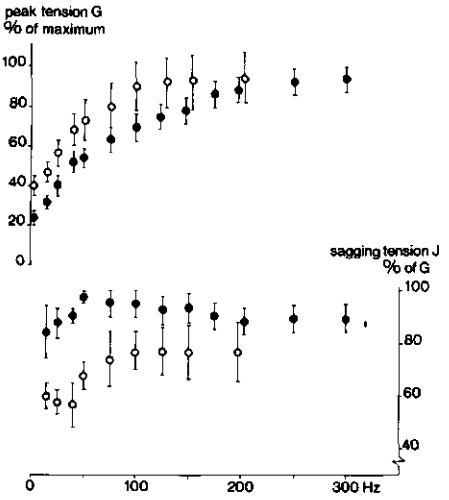

Fig. 7. Tension plotted against the stimulus frequency; ○: white fibres; ●: red fibres (mean ± SD; white $n=14$; red $n=12$). *Upper curves:* peak tetanic tension (G, see Fig. 5) as percentage of the maximal tetanic tension. *Lower curves:* sagging tension 1.5 s after the first stimulus (J, see Fig. 5) as percentage of the peak tension G. Sag is greater in white than in red fibres. It has a different frequency dependence in both types

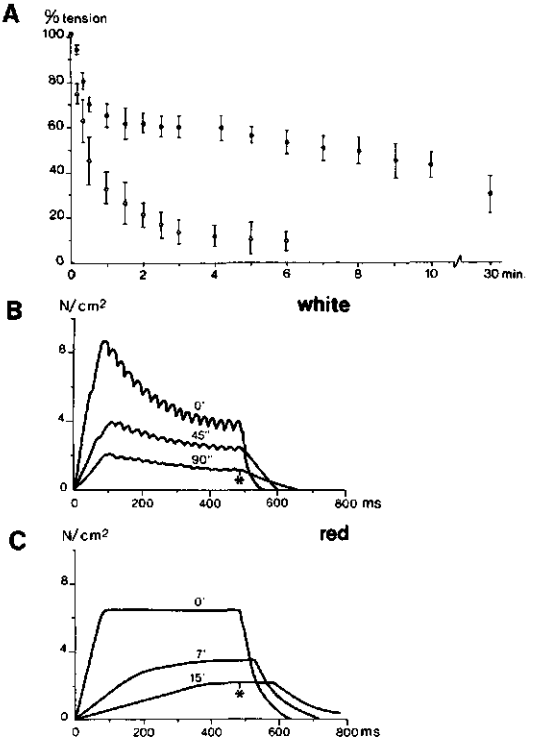

Fig. 8A–C. Fatigue after repeated 40 Hz tetani; ○: white fibres; ●: red fibres. **A** Maximal tensions of 40 Hz tetani, lasting 0.5 s, which were repeated every 2 s, plotted as percentage of the initial 40 Hz tetanic tension (mean ± SD, white $n=5$, red $n=4$). Red fibres have a better resistance to fatigue than white fibres. **B** First, 22nd, and 45th tetani of a white fibre bundle. **C** First, 210th and 450th tetani of a red fibre bundle. The decline in maximal tension of both types is accompanied by a decrease in the rate of tension development. In red fibres also a decrease in the time-to-peak-tension is seen. *: increasing the stimulus frequency at this point failed to increase the tension; this is in contrast to sagging tetani where increase of the stimulus frequency results in increase of tension

Epimuscular Stimulation

With epimuscular stimulation both fibre types also responded to a single stimulus with a twitch, but in contrast to nerve stimulation no supramaximal stimulus amplitude was found. In both fibre types the measured twitches and tetani increased with the increasing stimulus amplitude till the fibres coagulated. This phenomenon was more marked in red fibres than in white fibres (Table 3).

Another striking difference of direct stimulation as compared with stimulation by means of the nerve was the long relaxation time of both

Table 3. Epimuscular stimulation, twitch characteristics (see Fig. 4). Values are means ± S.D.

	White fibres		Red fibres	
	6 V–1 ms	12 V–1 ms ratio of values obtained by 6 V	6 V–1 ms	12 V–1 ms ratio of values obtained by 6 V
	($n=12$)	($n=4$)	($n=10$)	($n=4$)
A. Maximal twitch tension	1.6 ± 0.6 N/cm²	1.7	0.4 ± 0.2 N/cm²	2.5
B. Time-to-peak-tension	42 ± 6 ms	1.4	142 ± 90 ms	3.8
C. Half relaxation time	66 ± 33 ms	5.9	3.1 ± 2 s	15
D. Maximum positive slope	63 ± 33 N/cm² s	1.3	7 ± 4 N/cm² s	1.2
Relative positive slope	37 ± 9 s	0.8	17 ± 9 s^{-1}	0.5
E. Maximum negative slope	23 ± 14 N/cm² s	0.7	0.8 ± 0.9 N/cm² s	0.7
F. Twitch time	804 ± 350 ms	5.4	30 ± 21 s	3.0

twitches and tetani, especially in red fibres. This parameter increased with increasing stimulus amplitude; in red fibres relaxation times of about 30 sec could be measured. Relaxation of tetani showed a relatively steep phase followed by a phase of extremely slow decline of tension.

Tetani obtained by epimuscular stimulation also showed sag. After soaking the muscle preparations for about 15 min in frequently refreshed saline normal twitches and tetani could be obtained from these fibres by stimulation via the nerve.

Discussion

Both red and white fibres respond to a single stimulus with a twitch after neural as well as after epimuscular stimulation (Tables 1 and 3). Such twitch responses are known for white fibres (Johnston 1981) and for red fibres of the cod and the cuckoo ray (Johnston 1982). But red myotomal fibres of the tench (Barets 1961) and red fibres of the m. adductor operculi of *Tilapia mossambica* (Flitney and Johnston 1979) respond only to trains of stimuli. In view of these results it seems likely that red fibres of fishes are also physiologically a more heterogeneous group than has hitherto been appreciated.

Epimuscular Stimulation

With epimuscular stimulation no maximum tension can be obtained; increasing the stimulus amplitude results in an increase of tension which is accompanied by an increase in the tension rise time and a decrease of the relative slope of tension rise. This suggests that progressively greater parts of the stimulated fibres contribute to the tension. So it is unlikely that under these experimental conditions the fibres propagate action potentials over their entire length. Noteworthy is that in red fibres epimuscular stimulation results in a much slower and more incomplete development of tension than in white fibres.

In many teleosts including carp and perch, both red and white muscle fibres are multiterminally innervated (Bone 1978). Red muscle fibres become activated by summated junction potentials. These potentials reach plateau values which depend upon the stimulation frequency (Takeuchi 1959). Spikes are generally not observed in these fibres (Takeuchi 1959; Barets 1961; Hagiwara and Takahashi 1967). White fibres also respond to stimulation with graded junction potentials, which may result in contraction (Takeuchi 1959; Hudson 1969). At reaching a critical level summated junction potentials of white fibres result in the generation of a spike. In some fishes these are observed to be 'abortive spikes' having only a small overshoot or no overshoot at all (Takeuchi 1959; Barets 1961; Hagiwara and Takahashi 1967; Hidaka and Toida 1969). In other fishes propagated action potentials having a distinct overshoot are described (Hudson 1969; Gainer and Klancher 1965). We found that both red and white muscle fibres of the carp are only partially activated by local epimuscular stimulation.

Multiterminally innervated muscle fibres of fishes are probably also polyneurally innervated (Bone 1978). It is therefore possible that, as has been suggested on the basis of electromyographical recordings by Bone et al. (1978) and Johnston and Moon (1980a, b), these fibres are also locally activated in vivo.

Epimuscularly stimulated muscle fibres of the carp, especially red fibres, show a very slow decline of tension. This suggests a partial deactivation of the relaxation mechanism. This deactivation is reversible as after recovery normal results can be obtained from these fibres by stimulation via the nerve. From these results it is apparent that epimuscular stimulation is an unsatisfactory technique for the study of the mechanical properties of fish muscle fibres.

Nerve Stimulation

Twitch Contraction Time. We found tension rise times of about 30 ms for white fibres and of 60 ms for red fibres, both with very small variations. As the contraction velocity of fish muscle depends upon the size of the fish and the experimental temperature (Wardle 1975) only qualitative comparison with the literature is possible. Described twitch contraction times for white fibres, excepting the very fast swimbladder muscles, range from 12 ms for squirrelfishes (Gainer et al. 1965) to about 50 ms for the cuckoo ray (Johnston 1980). The twitch contraction time of 40 ms found for a red pectoral fin muscle of *Carassius auratus* (Yamamoto 1972) may represent the velocity of a mixture of slow and fast fibres as histochemical observations on pectoral fin muscles of carp (Akster and Woittiez, unpublished results) reveal that these muscles contain an appreciable amount of pink (aerobic, fast) fibres. That white fibres have a higher contraction velocity than red fibres is not unexpected. White fibres are known to be used in fast, and red fibres in slow movements (Bone 1966; Johnston et al. 1977; Akster and Osse 1978).

Moreover the myofibrillar ATPase activity, which is proportional to the intrinsic contraction velocity (Bárány 1967), is known to be higher in white than in red fibres (Nag 1972; Johnston et al. 1977; van Raamsdonk et al. 1980).

Maximal Tetanic Tension. We found similar tetanic tensions per cross section area for both muscle types, about 12 N/cm^2. This is much higher than the values obtained by epimuscular end-to-end stimulation of white myotomal fibres of cod and cuckoo ray (0.5 and 1.4 N/cm^2, respectively; Johnston 1980). It is also higher than the values obtained for cod fibres which were epimuscularly stimulated over their entire length (about 9 N/cm^2 for white fibres and about 3 N/cm^2 for red fibres; Johnston 1982). For white fibres it is lower than the tensions obtained by adding ATP and calcium to skinned white fibres of cod and cuckoo ray (about 18 N/cm^2; Johnston 1982). For red fibres it is slightly higher than the maximal value for skinned red fibres of cod and dogfish (about 8.5 N/cm^2; Johnston 1982). These tensions are in the same order of magnitude as the maximum tetanic tensions per cross section area of contractile material in mammalian fibres (about 20 N/cm^2, both for fast and slow fibres; Close 1972).

Rate of Tension Development. The maximum rate of tension development in white fibres is independent of the stimulus frequency. It is similar in a twitch and in tetani of different frequency (Table 2, Fig. 6D). In red fibres the maximum rate of tension rise increases at stimulation frequencies exeeding 100 Hz. At maximum tetanic tension this slope is 1.33 times that of a twitch (Table 2, Fig. 6E). This difference in tension rise is probably one of the causes of the low ratio of twitch tension to maximum tetanic tension in red fibres (0.27) as compared with that in white fibres (0.42). Carp red fibres generate no action potentials. It is possible that fibre parts which are not in the immediate vicinity of endplates are not activated after a single stimulus but only at depolarisation values obtained by the summation of several junction potentials. This may cause an increase in the rate of tension rise at high stimulation frequencies.

Slow and fast twitch fibres of mammals have similar maximal tetanic tensions and similar ratio's of twitch tension to maximal tetanic tension (about 0.25; Close 1972). Slow as well as fast fibres of mammalian limb muscles are known to generate action potentials but their rate of tension development is dependent upon the stimulus frequency (Buller and Lewis 1965). Mechanical activation of these fibres after a single stimulus is incomplete (Close 1972; Ranatunga 1978). This is attributed to incomplete spread of activation inward to the fibre's core (Wallinga-de Jonge et al. 1980). In red fibres of fishes, sarcomeres of superficially situated myofibrils can also be expected to become more easily activated than those in the fibre's core.

Decline of Tension. This is more rapid in white than in red fibres of the carp. Tension decline is proportional to the velocity of removal of calcium from the myofibrils and its uptake by the sarcoplasmic reticulum (SR). In this process a calcium-binding protein, parvalbumin, which can act as a calcium-shuttle between the myofibrils and the SR probably plays an important role (Gerday and Gillis 1976). Generally parvalbumin is present in large amounts in white muscle fibres and in small amounts in red fibres (Hamoir et al. 1981). Also white fibres of fishes have been described to have more and faster working SR than red fibres (Nag 1972; Kilarski 1973; McArdle and Johnston 1981). The differences in tension decline between red and white fibres of the carp is consistent with these biochemical and ultrastructural results.

Sag, the decline of tension during a tetanus shortly after the maximum tension is reached, is more pronounced in white than in red fibres. The frequency dependence of sag also differs in both types (Fig. 7). In each type, sag measured 1.5 s after the first stimulus, is maximal at a pulse interval similar to the twitch contraction time. This is also found for mammalian muscle (Burke et al. 1973). It is improbable that sag is due to fatigue as the tension of a sagging tetanus can be raised by increasing the stimulus frequency. This is not possible in fibres which are fatigued by repeated tetanization. Because sag also occurs in epimuscular stimulation it is not very likely that it is due to failure in neurotransmission. Burke et al. (1976) suggested that sag may be caused by a decrease in the duration and/or the intensity of the active state. This would mean that during a tetanus the amount of calcium available for binding to troponin diminishes. Somlyo et al. (1980) describe that in frog semitendinosus muscle by the end of a 1.2 s tetanus 60% of the initial calcium content is released from the terminal cisternae of the sarcoplasmic reticulum. They suggest that by then most of the released calcium is bound to parvalbumin. This is consistent with the progressive decrease in calcium release during successive twitches (Blinks et al. 1978). It is possible that this decrease in the output of calcium per stimulus results in the onset of sag. The increase in tension obtained by raising

the stimulus frequency during a sagging tetanus may be attributed to the increase in the frequency of calcium outputs. But the observation that in white fibres sag is greater in a 40 Hz tetanus than in a 100 Hz tetanus (see Fig. 7) is less easily explained. Blinks et al. (1978) found no obvious correlation between the fading (sag) of a tetanus and the uptake of calcium by the sarcoplasmic reticulum as measured with aequorin fluorescence. They suggest that fading might reflect loss of calcium from the muscle fibre. For understanding the phenomenon 'sag' more knowledge of the calcium movements in muscle fibres during a tetanus is required.

Resistance to Fatigue. This was studied by comparing the peak tensions of tetani obtained by 40 Hz pulse trains of 500 ms which were repeated every 2 s. This pattern of stimulation was chosen to minimize effects due to sag or failure of neurotransmission and because it is probably within the physiological range of stimulus frequencies (Ballintijn, personal communication). White fibres are very sensitive to fatigue (Fig. 8); their peak tension shows a 50% drop after approximately 20 tetani; within 5 min a 90% drop in tension occurs. Similar results were obtained by Wardle (1975) with white myotomal fibres of *Pleuronectus platessa*. Red fibres are much more resistant to fatigue than white fibres (Fig. 8), but a tension drop of 50% was reached within 10 min, and after 30 min approximately a third of the tension is left. Swimming movements executed by red fibres can be sustained for hours (Bone 1978; Johnston and Moon 1980a, b). It is however possible that in such cases not all red fibres are active at the same time. Moreover in polyneurally innervated fibres even different fibre parts may be active at different times. It is also possible that part of the measured decline in tension is caused by the fact that during the experiments the saline could not be refreshed, whereas in vivo red fibres have an abundant blood supply.

In both fibre types increase of fatigue is accompanied by a decrease in the maximum rates of tension development and of tension decline. Burke et al. (1973) made similar observations for mammalian muscles. In red fibres also an increase in contraction time was seen which was not observed for white fibres. It is possible that this is due to an intrinsic difference between the fibre types but as the red part of the m. hyohyoideus contains two red subtypes (Akster, in preparation) it is also possible that this phenomenon is caused by one of the subtypes having a greater resistance to fatigue and a lower contraction velocity than the other.

The significance of the variety of fibre types present in fish muscle will become more clear when functional data for all these types are available.

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Recently, the contractile properties of isometric twitches and tetani of fast (pink) and slow (a mixture of two red types) muscle fibres of the m.hyohyoideus of *Cyprinus carpio* have been described by Granzier et al. (1983). This allows a comparison of these properties with the relative volumes of mitochondria, myofibrils and membrane systems, the length of T-SR contact and the length of the sarcomere components measured in the present study.

In a previous study (Akster, 1983), small- and large pink fibres of the carp were found to differ in reactivity with an antiserum against myosin, in the pH stability of their myosin-ATPase and in succinate dehydrogenase activity. Therefore, in the present study, these fibres were sampled and investigated separately. To obtain a survey of the crucial differences between the five fibre types described in carp muscle (Akster, 1983), the extent of contact between the T-system and the SR was also measured in red b fibres and in white fibres of other muscles.

MATERIAL AND METHODS

Muscle fibres

Carp, of 17 - 27 cm standard length, bred in the laboratory at 20° C, and fed on commercial fish food (trouvit pellets) were used. Pink fibres were taken from the dorso-caudal part of the m.hyohyoideus superior (fig.1a). Red fibres, a mixture of the types red a and red b, were taken from the red part of the m.hyohyoideus inferior (fig. 1a). From these fibre types ultrathin cross- and longitudinal-sections were studied. Type red b fibres were taken from the medial rim of the dorso-caudal part of the m.hyohyoideus superior (fig. 1a) and white fibres were taken from the axial muscles (fig. 1b). These fibre types were studied in longitudinal sections only. Of all these types, fibres of at least five fishes were studied.

Electron-microscopy

The opercular bones with the m.hyohyoideus were, slightly stretched, pinned on a layer of paraplast and immersed in Karnovsky's fixative. The same fixative was injected into the axial muscle directly caudal to the anus. After fixation at room temperature for about one hour, the investigated fibre bundles were dissected, immersed for 12-19 hours in fresh Karnovsky's fixative, postfixed for two hours in osmiumtetroxide, dehydrated and embedded in an epon mixture. Ultrathin sections were cut with a diamond

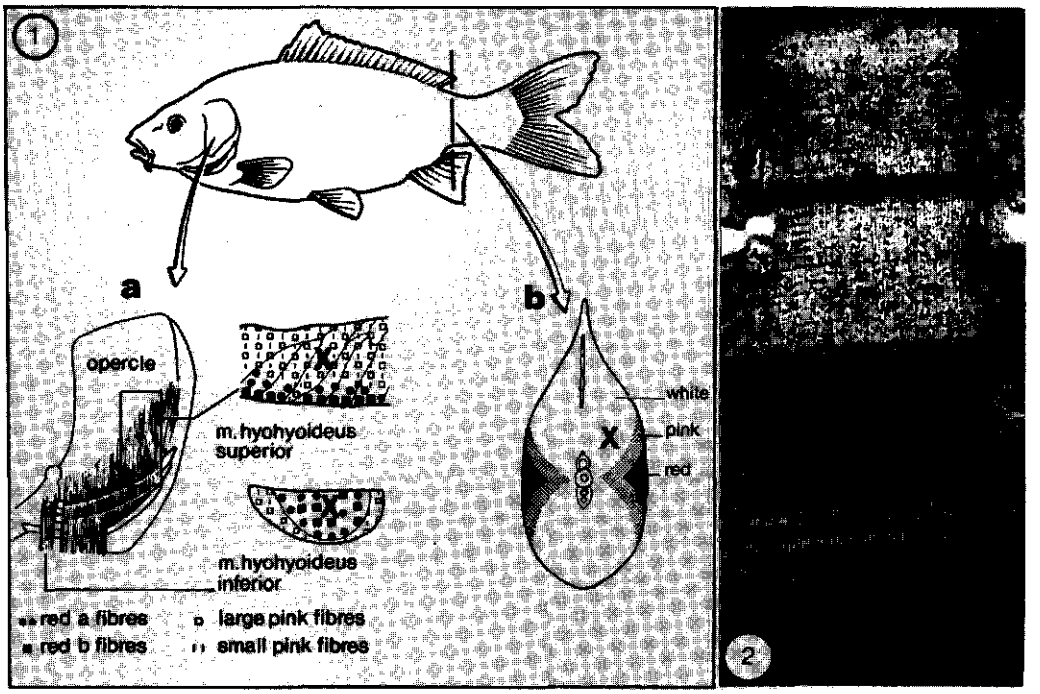

Fig. 1. Localization of the investigated muscle fibres; a. medial view of the right opercular bones with the m.hyohyoideus and the position of the investigate fibres (X). b. axial muscles.

Fig. 2. Red fibre, longitudinal section 44000x, lines: striation in the I band caused by the troponin periodicity.

knife on a Reichert OMU II ultramicrotome, stained with uranylacetate and leadcitrate, and studied on a Philips 400 transmission electron-microscope (Electron-microscopy unit of the institute of Agricultural Engineering, Wageningen).

In order to distinguish small- and large pink fibres in longitudinal sections, semi-thin cross-sections were made of the re-oriented blocks before and after cutting the longitudinal ultrathin sections. The fibres present in the longitudinal sections were located in camera lucida drawings of the semi-thin cross sections and their areas were measured with a datatablet (Summagraphic Supergrid). Small pink fibres have cross sectional areas below 500 μm^2 (mean $\pm$ sem:284 $\pm$ 28 μm^2); large pink fibres have areas above 500 μm^2 (mean $\pm$ sem: 1004 $\pm$ 111 μm^2). The mean area of all pink fibres, present in the semi-thin cross sections (532 μm^2), was also determined with the datatablet. For parameters that differed in the small pink fibres and the large pink fibres, a mean value for pink fibres was calculated by interpolating to this mean fibre size.

Morphometry

Relative volumes and surface areas were measured as described by Weibel and Bolender (1973) and Eisenberg et al. (1974) with quadratic test lattices.

Relative volumes, expressed as a percentage, were derived from $\frac{V_v}{V_t} = \frac{P_v}{P_t} \times 100$, in which V_v is the volume of the measured organelle and P_v the number of test points falling on this organelle. V_t is the total volume of tissue and P_t is the total number of test points falling on the tissue. The relative volumes of the subsarcolemmal mitochondria, of the nuclei, and of the myofibrillar core were measured in cross sections of whole fibres (or, in case of very large pink fibres, half a fibre), with a test lattice space of 1.4 µm. The relative volume of intermyofibrillar mitochondria was measured in two detail micrographs of the myofibrillar core with a lattice space of 0.84 µm and 169 test points. From these values the total relative volumes of mitochondria, of myofibrils and of the subsarcolemmal cytoplasm (see fig. 7) were calculated as percentages of the fibre volume.

The length of the myofibril boundary per unit cross sectional area ($\mu/\mu^2 = \mu^{-1}$) which is inversely proportional to the myofibril girth, was derived from $M_b = \frac{\pi I}{2L_t}$, in which L_t is the total line length of the test grid (2 x 156 x 0.84 µm) and I the number of intersections of the test grid with the measured structure.

Measurements of the membrane systems (T system + sarcoplasmic reticulum) were made on two micrographs per fibre from longitudinal sections with a test lattice space of 0.4 µm and 100 test points. To compensate for the

distinct orientation of the muscle fibre components, the test lattice was placed at an angle of 19° or 71° to the fibre axis (Sitte, 1967; Eisenberg et al. 1974).

The relative membrane area (S_v) was calculated from $S_v = \frac{2I}{L_t}$.

L_t (2 x 90 x 0.4 μm) and I have been defined above. A correction for the partial anisotropy of the SR tubules, as determined by Eisenberg et al. (1974), was not applied; this may result in an underestimation of the values measured in the present study of less than 6 %. However, in a study on perch muscle (Akster et al., in preparation), the relative volumes and surface areas of the SR,measured in cross sections, were about 80 % of those measured in longitudinal sections. This difference is probably caused by an over-representation of the SR tubules in the longitudinal sections as a result of the section thickness effect (Holmes effect), which depends on the ratio of the section thickness to the diameter of the measured structures. As in carp muscle, larger SR tubules were found than in perch muscle (minor axis 55 $\pm$ 12 nm and 35 $\pm$ 7 nm, mean $\pm$ SD, n = 75, respectively), overestimation, due to the section thickness effect, will be smaller for carp muscle than for perch muscle. Since, in the present study, it is not known to what extent the mentioned effects cancel and since the relative difference between the fibre types will not be influenced by an correction factor, no correction was applied.

The relative line length of the T-SR contact per unit volume of myofibrillar material ($\mu/\mu^{-3} = \mu^{-2}$) was determined by counting the number of contact profiles per μm^2 myofibrillar material (Eisenberg and Salmons, 1983). Diads, T-system profiles making only contact with the SR on one side, were counted as one contact. Triads, T-system profiles making contact with the SR on two sides (figs. 5 and 6) were counted as two contacts. In order to obtain a value that is more closely related to the contraction velocity, all parameters concerning the membrane systems are given per volume unit myofibrillar core corrected for mitochondria.

For measuring the Z-line thickness and the lengths of the actin- and myosin filaments, the cross striation in the I band (fig. 2), caused by the 39 nm peridicity of the troponin complexes on the actin filament, was used (Page and Huxley, 1963). The combined effects of magnification and shrinkage could be calculated from the measured distance between the cross striations, so that the natural length of the filaments could be obtained.

table 1. Fibre composition, mean ± sem.

	n	a range of cross section. areas	b total mitochondria	c intermyofibrillar mitochondria	d myofibrils	e myofibrillar boundary	f nuclei	g subsarc. cytoplasm
		μm^2	vol.%	vol.%++	vol.%	μm^{-1}++	vol.%	vol.%
Pink	32	86 - 2160	4.3 ± 0.4+	0.4 ± 0.2	84.1 ± 0.9+	1.3 ± 0.03	0.4 ± 0.2	11.2 ± 1.0+
Large pink	15	500 - 2160	2.2 ± 0.3	0.4 ± 0.1	91.4 ± 0.8	1.3 ± 0.04	0.3 ± 0.1	6.1 ± 0.8
Small pink	17	86 - 500	5.7 ± 0.6	0.4 ± 0.2	79.8 ± 1.0	1.3 ± 0.03	0.4 ± 0.2	14.1 ± 1.1
Red	30	58 - 1089	24.8 ± 1.6	6.7 ± 0.9	57.4 ± 2.7	1.5 ± 0.04	0.9 ± 0.4	16.7 ± 1.5

+ calculated by interpolating the values for small- and large pink fibres to the mean size for pink fibres.

++ calculated per volume of the myofibrillar core; the other values are calculated per fibre volume.

Statistics

Significances of the measured differences in myofibrillar boundary, Z-line thickness, T-SR contact and the relative volumes and areas of membrane systems were calculated by means of Students T test or, in case of unequal variances, with an approximate T test (Sokal and Rohlf, 1965).

RESULTS

Fibre composition (table 1)

The pink fibres (fig. 3) were very variable in size; their mean cross sectional area was 532 μm^2. The size of the red fibres (fig. 4) was less variable; their mean cross sectional area was 376 μm^2 (for the range of fibre cross sectional areas, see table 1a).

Red fibres had higher relative volumes of subsarcolemmal and intermyofibrillar mitochondria (tables 1b and 1c) and of subsarcolemmal cytoplasm (1g) than pink fibres. They had a lower relative volume of myofibrils (table 1d) and they contained more glycogen (figs. 5 and 6). The myofibrillar boundary (table 1e) was slightly longer in red fibres, which means that in this type slightly smaller myofibrils were found ($P<0.05$).

Small pink fibres had a higher relative volume of subsarcolemmal mitochondria than large pink fibres; they had a similar relative volume of intermyofibrillar mitochondria (tables 1b and 1c). They had a lower relative volume of myofibrils (table 1d) and more subsarcolemmal cytoplasm (table 1g) than large pink fibres.
Differences in the relative volumes of nuclei (table f) were not significant.

Myofilaments (table 2)

table 2. Sarcomere- and filament lengths and Z-line thickness, mean ± sem.

		sarcomere	actin	myosin	Z line
	n	µm	µm	µm	nm
Pink	29	2.59 ± 0.10	0.95 ± 0.03	1.73 ± 0.02	98 ± 4
Large pink	14	2.62 ± 0.09	0.95 ± 0.03	1.74 ± 0.03	98 ± 6
Small pink	15	2.57 ± 0.10	0.95 ± 0.02	1.73 ± 0.02	97 ± 2
Red	19	2.78 ± 0.13	0.95 ± 0.03	1.72 ± 0.03	109 ± 3

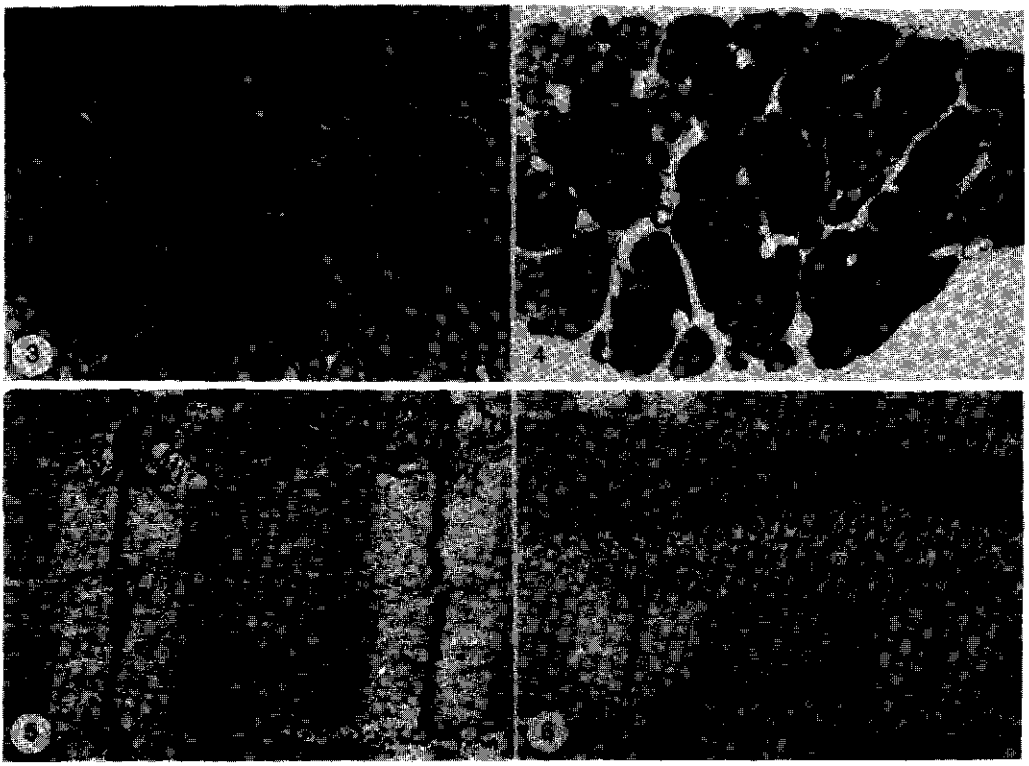

Fig. 3. Semi-thin cross section of the pink part of the m.hyohyoideus superior. Note the variation in fibre size. 300x.

Fig. 4. Semi-thin cross section of a red fibre bundle of the m.hyohyoideus inferior. The fibres are surrounded by capillaries (c) and contain many mitochondria, visible as dark dots (arrows). 300 x.

Fig. 5. Small pink fibre, longitudinal section 25000x. arrows: T-SR contacts.

Fig. 6. Red fibre, longitudinal section 25000x. M: mitochondrion, G: glycogen, arrows: T-SR contacts.

Actin filament length as well as myosin filament length were similar in red and pink fibres. Sarcomere shortening was variable in all types, but generally red fibres were less shortened than pink fibres. Red fibres had thicker Z-lines than pink fibres ($P<0.05$).

Membrane systems (tables 3 and 4)

In all investigated fibre types the T-system was found at the level of the Z-line (figs. 5 and 6). The only significant difference between the membrane systems of red and pink fibres was the length of T-SR contact: pink fibres had a greater length of T-SR contact than red fibres (table 3a). Therefore this parameter was also measured in other fibre types. White fibres had a greater length of T-SR contact than pink fibres, and red b fibres had a smaller length of T-SR contact than the mixture of red a and red b fibres.

table 3. Membrane systems, (SR + T system) mean ± sem.

	n	a length of T-SR contact μm^{-2}	b relative volume vol.%	c relative surface area μm^{-1}
Axial White	28	0.77 ± 0.04	-	-
Pink	32	0.66 ± 0.04^{+}	4.4 ± 0.4^{+}	2.8 ± 0.2^{+}
Large pink	17	0.66 ± 0.03	5.9 ± 0.5	3.0 ± 0.2
Small pink	15	0.65 ± 0.05	3.7 ± 0.4	2.6 ± 0.2
Red (a+b)	40	0.53 ± 0.03	5.4 ± 0.5	3.2 ± 0.2
Red b	35	0.44 ± 0.06	-	-

Calculated per unit volume of the myofibrillar core, corrected for mitochondria.

$^{+}$Calculated by interpolating the values for small- and large pink fibres to the mean fibre size.

Significance of differences in T-SR contact: white-pink $P<0.01$; pink-red $P<0.05$; red (a+b)-red b $P<0.05$.
Significance of differences in relative volume: small pink-large pink $P<0.05$.
No significant differences in relative surface area were found.

Small pink fibres had a significantly lower relative volume of membrane systems (table 3b) than large pink fibres, but they had a similar length of T-SR contact (table 3a).

The values of the relative volumes and areas of membrane systems in the red fibres were slightly higher than the mean value found for the pink fibres

(table 3b). This is probably caused by the presence of several red fibres with greatly swollen terminal cisternae, which gave cross sections of these fibres a perforated appearance (fig. 7).

Fig. 7. Red fibres and part of a pink fibre (below right) cross section 6800x. MY: myofibrils, IM: intermyofibrillar mitochondria, SM: subsarcolemmal mitochondria, N: nucleus, P: subsarcolemmal cytoplasm, outside the myofibrillar core, C: capillary. One of the red fibres, (upper left) has a perforated appearance, due to the greatly distended terminal cisternae of the SR (arrows).

Comparison of the length of T-SR contact of red fibres with a relative volume of membrane systems above the mean volume with that of red fibres with a relative volume of membrane systems below it, showed for both groups the same extent of T-SR contact (table 4). This indicates that the swelling of the terminal cisternae is not related to the extent of T-S contact.

table 4. Length of T-SR contact in red fibres with high (above the mean) and with low (below the mean) relative volumes of membrane systems.

	n	relative volume vol.%	relative surface area μm^{-1}	length of T-SR contact μm^{-2}
low	24	3.5 ± 0.2	3.0 ± 0.2	0.53 ± 0.03
high	16	8.7 ± 0.8	3.6 ± 0.2	0.53 ± 0.04

DISCUSSION

In this study, several ultrastructural parameters have been measured, in order to establish to what extent they correspond with the contractile properties measured by Granzier et al. (1983).
Significant differences between red- en pink fibres were found in the length of T-SR contact, in Z-line thickness and in the relative volumes of myofibrils and mitochondria. Small- and large pink fibres differed in the relative volumes of membrane systems and of subsarcolemmal mitochondria. The relation of these parameters with the contractile properties of the fibre types will be discussed.

T-SR contact

The extent of T-SR contact showed significant differences between four main muscle fibre types of the carp. This agrees with observations on perch (*Perca fluviatilis L.*) muscle (Akster, 1981; Akster et al. in preparation); also in this fish, fibres with short contraction times have a more extensive T-SR contact than slower fibres. Since the spread of excitation from the T-system to the SR is most probably effected at the T-SR junction, involving structural changes of this junction (Eisenberg and Eisenberg, 1982), it is very likely that the extent of T-SR contact influences the rate of calcium release and thereby the rate of tension development. Thus the length of T-SR contact is probably a useful indication for differences in contraction velocity.

The muscle fibres investigated in this study had the T-system at the Z-line. Fibres with the T-system at the junction of A band and I band, as described by Zawadowska and Kilarski, (1983), for the eye muscles of *Cyprinus carpio*, were not found. As, except for very fast muscle fibres, the position of the T-system is related to the actin filament length (Page, 1968; Akster, 1981), the position of the T-system at the Z-line corresponds well with the relatively short actin filament lengths measured in the present study.

Volume and surface area of membrane systems

The long half-relaxation time of red fibres (more than twice that of pink fibres, Granzier et al., 1983) is not accompanied by a low relative volume or surface area of the membrane systems. The, unexpected, high relative volume of the membrane systems in the red fibres is very probably caused by the presence of swollen terminal cisternae in some of these fibres. As this was only found in a minority of the fibres, while adjacent fibres had a quite normal appearance (fig. 7), it can not be attributed to a fixation artifact. A similar swelling of the SR was found by Eisenberg and Salmons (1981) in muscle fibres of rabbits that were in the early stages of being turned from fast into slow fibres by continuous stimulation.

That small pink fibres have a lower relative volume of membrane systems than large pink fibres was also described by Egginton and Johnston (1982) for the eel, *Anguilla anguilla L.* In the present study, however, small- and large pink fibres appeared to have similar extents of T-SR contact so that differences in contraction velocity are not expected.

Z-line thickness

That slow (or red) fibres have thicker Z-lines than fast (white or pink) fibres, as was found in the present study, has also been described for other fishes (Patterson and Goldspink, 1972; Slinde and Kryvi, 1980; Kilarski and Kozlowska, 1983; Te Kronnie et al., 1983). However, the functional implications of differences in Z-line thickness are still unknown. This makes it a less satisfactory parameter for the distinction of muscle fibre types.

Myofibrils

Red fibres have a relative volume of myofibrils that is about two-thirds of that of pink fibres, and red fibre bundles contain more connective tissue and capillaries than bundles of pink fibres (figs. 3 and 4). So they can be

expected to reach less than two-thirds of the maximal tension of pink fibres. The measured maximal isometric tetanic tension of red fibre bundles, however, is much closer to that of pink fibres (11N/cm^2 and 12 N/cm^2, respectively, Granzier et al., 1983); than is expected. A comparable result was found for red and pink muscle fibres of the perch, which also have similar maximal tetanic tensions (16 N/cm^2 and 17 N/cm^2, respectively, Akster et al., in preparation). This similarity contrasts to data of Altringham and Johnston (1982) who, for fast and slow muscle fibres of the cod (*Gadus morhua*) and the dogfish (*Scyliorhinus canicula*) found that the difference in maximal tension was greater than would be expected on the basis of the difference in the relative volumes of myofibrils. Our results suggest that red myofibrils of carp and perch muscle may generate a higher force per unit cross sectional area than those of fast fibres of the same species.

Mitochondria

Red fibres have more glycogen and a higher relative volume of mitochondria than pink fibres. This corresponds with a better endurance of the red fibres (Granzier et al., 1983).

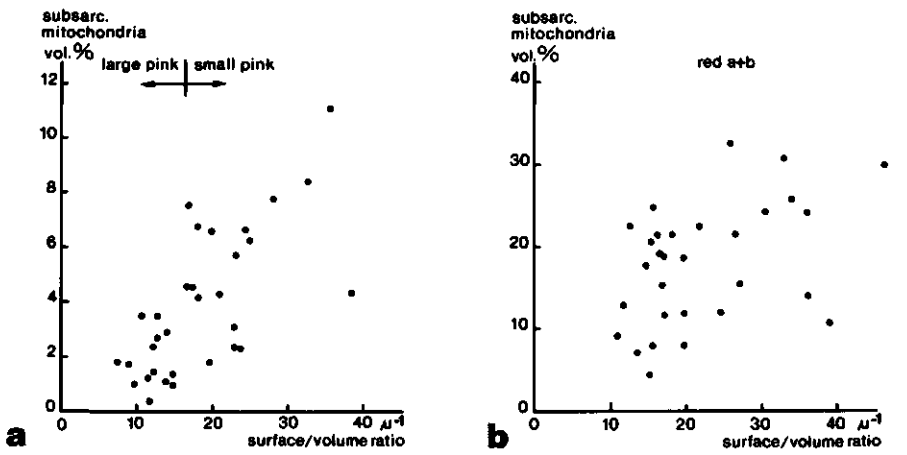

Fig. 8. Diagrams of the volume percentage of subsarcolemmal mitochondria plotted against the surface/volume ratio of the fibres.
a. pink fibres r = 0.67, n = 32. b. red fibres r = 0.43, n = 30.

The difference in the relative volume of mitochondria between large- and small pink fibres, which corresponds to a histochemical difference in succinate dehydrogenase activity (Akster, 1983), concerns only subsarcolemmal mitochondria; the relative volume of intermyofibrillar mitochondria does not differ.
On the basis of biochemical (Kubista et al., 1978) and morphological (Muller, 1980) data, the subsarcolemmal mitochondria are thought to provide the energy needed for active transport through the sarcolemma; the intermyofibrillar mitochondria are supposed to provide the energy needed for contraction. According to this hypothesis,the higher relative volume of subsarcolemmal mitochondria found in small fibres may reflect their higher surface to volume ratio.
A diagram, in which the relative volume of mitochondria is plotted against this ratio (fig. 8a), indeed shows this scale effect. It shows that about half of the variance in the relative volume of subsarcolemmal mitochondria can be related to this size effect ($r^2 = 0.45$).
A similar diagram for the red fibres (fig. 8b) shows that in this group the variance in the relative volume of subsarcolemmal mitochondria is less related to size differences.

Fibre types

On the basis of histochemistry, four main fibre types: white, pink, red a (red in the perch) and red b (deep red in the perch) were distinguished in head muscle and axial muscle of perch and carp (Akster and Osse, 1978; Akster and Sibbing, 1982; Akster, 1983). In addition to this histochemical conformity, also an ultrastructural conformity is found. In both fishes, a gradual decrease in the extent of T-SR contact occurs: from a high value in the white fibres, through lower values in the pink and then in the red fibres, to low values in the deep red (red b) fibres. This suggests a gradual decrease in contraction velocity in the same order.

A striking difference between carp and perch muscle is the paucity of white fibres in the head muscles of the carp (Akster and Sibbing, 1982). White fibres from the axial muscles of the carp have a less extensive T-SR contact ($0.77\ \mu m^{-2}$) than white fibres of the axial muscles of the perch ($0.92\ \mu m^{-2}$, Akster et al, in preparation), which suggests that those of the carp are slower. This difference would be in agreement with ecological differences between both fishes. The perch, a predator, which chases and captures its prey with sudden powerful movements, has more need for a fast type of

muscle fibre than the carp, an omnivorous euryphagous fish (Sibbing, 1982), for which feeding is a more permanent and more leisurely activity.

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VI

A COMPARISON OF QUANTITATIVE ULTRASTRUCTURAL- AND CONTRACTILE CHARACTERISTICS OF MUSCLE FIBRE TYPES OF THE PERCH, PERCA FLUVIATILIS L.

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SUMMARY

Quantitative ultrastructural and physiological parameters were investigated in three types of muscle fibres of *Perca fluviatilis* L.: white fibres of the m.levator operculi anterior, pink (intermediate) fibres of the m.hyohyoideus and deep red fibres of the m.levator operculi anterior. Times to peak tension and half relaxation times of isometric twitches increased in the mentioned order. The extent of contact between the T system and the sarcoplasmic reticulum and the relative volume and surface area of the terminal cisternae showed an inverse relation with the time to peak tension of the twitch. The maximal isometric tetanic force per unit cross sectional area was similar for all three investigated types. The inverse relation between the time to peak tension of the twitch and the relative length of contact between T system and SR is in agreement with data obtained for fast- and slow twitch muscle fibres of the carp, *Cyprinus carpio* L.

INTRODUCTION

Muscles of fishes, especially head muscles, participate in a variety of movements that impose different, often conflicting, demands upon them. These muscles contain a variety of fibre types, which, by means of electromyography, have been shown to be employed during different movements (Johnston et al., 1977; Bone et al., 1978; Akster and Osse, 1978; Barends, 1978; Vandewalle et al., 1983). It is to be expected that this specialization will be reflected in the ultrastructural- and contractile properties of these fibre types; the relations between these properties are investigated in the present study.

Quantitative ultrastructural data on membrane systems of fish muscle fibre types have been published before (Nag, 1972; Kilarski, 1972; Patterson and Goldspink, 1972; Korneliussen and Nicolaysen, 1975; Kryvi, 1978; Quaglia, 1980; Akster, 1981; Egginton and Johnston, 1982); but the described differences in the relative volume of the sarcoplasmic reticulum are not consistent and comparison between structural and physiological data has seldomly been attempted. Data of times to peak tension of the twitches of white, or fast, muscle fibres of fishes are scarce (Gainer et al., 1965; Johnston, 1980, 1982; Granzier et al., 1983), and those of red, or slow, muscle fibres of fishes have only been described by Johnston (1981,1982) and Granzier et al. (1982,1983).

The fibre types selected for this study, are white and deep red fibres of the m.levator operculi anterior (LOPA) of the perch (fig. 1), which have been described by Akster and Osse (1978) and Akster (1981), and fibres of the m.hyohyoideus (HH), which, on the basis of data obtained in this study, can be classified as pink or intermediate fibres.

MATERIAL AND METHODS

Perch (*Perca fluviatilis* L.), measuring 15 - 25 cm, were obtained from commercial fishermen and kept in tanks at a temperature of 15 - 20°C, until needed. The fishes were anaesthetized with 0.1% MS222 (Sandoz) and the opercular bones (fig. 1) with the m.levator operculi anterior (LOPA) and the m.hyohyoideus (HH) were rapidly dissected. For white LOPA fibres, the largest fibres from the medial part of this muscle were selected. Deep red fibres were taken from the lateral part of the LOPA, which contains only this type (Akster and Osse, 1978).
The HH fibres were taken from the dorso-caudal part of this muscle (fig. 1).

Ultrastructural measurements

Small bundles of deep red LOPA fibres and small bundles of HH fibres of five fishes were mounted between steel hooks and immersed for 3 hours in 6% glutaraldehyde in 0.1M cacodylate buffer at pH 7.3. After postfixation in a solution of 1% OsO_4 and 50 mM ferrocyanide in cacodylate buffer, the tissue was dehydrated and embedded in an epon mixture. Ultrathin sections were cut with a diamond knife on a Reichert OMU II ultramicrotome, stained with uranylacetate and leadcitrate and studied on a Philips 400 transmission electronmicroscope. (Electron-microscopy unit of the Institute of Agricultural Engineering, Wageningen).

For measurements on the membrane systems of white fibres of the LOPA, the material described by Akster (1981) was used. Data obtained from this material for deep red fibres of the LOPA were similar to data obtained for this fibre

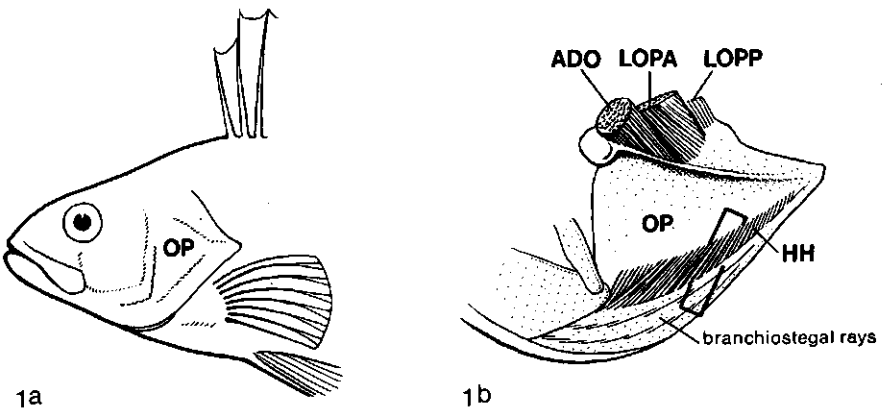

Fig. 1. Perch head (1a) and medial view of the opercular bones (OP) with the studied muscles (1b). ADO: m. adductor operculi; LOPA: m.levator operculi anterior; LOPP: m.levator operculi posterior; HH: m.hyohyoideus; rectangle: position of the studied fibres of the m.hyohyoideus.

type from the five fishes used in the present study (table 2). Thus it appears that data from both sets of material are comparable.

Stereological measurements were performed as described by Weibel and Bolender (1973) and by Eisenberg et al. (1974) with quadratic test lattices of 121 test points. Per fibre two micrographs of longitudinal sections were used. The test lattice space was 0.23 µm for the deep red fibres of the LOPA and the fibres of the hyohyoideus and 0.29 µm for the white fibres of the LOPA. To compensate for the fact that muscle fibre components have a distinct orientation, the test lattice was placed at an angle of 19° or 71° to the fibre axis (Eisenberg et al., 1974). Relative volumes, expressed as percentages, were derived from $\frac{V_v}{V_t} = \frac{P_v}{P_t} \times 100$, in which V_v is the volume of the measured organelle, P_v is the number of test points falling on this organelle, V_t is the total volume of tissue and P_t is the total number of test points falling on the tissue. The area of terminal cisternae membrane per unit volume of tissue, S_{vt}, is derived from $S_{vt} = \frac{2I}{L_t}$, in which L_t is the total line length of the test grid and I is the number of intersections of the test grid with the boundaries of the membrane system. The surface area of the longitudinal tubules

was derived from $S_{vl} = \frac{1}{2}\left(\frac{\pi}{\sqrt{2}} + 2\right)\frac{I}{L_t}$ which assumes a partial orientation, parallel to the plane of section, of these structures (Eisenberg et al., 1974).

The relative line length of the contact between the T-system and the SR (T-SR contact) per unit volume of tissue was determined by counting the number of T-SR contacts (see fig. 2) per μm^2 myofibrillar material (Eisenberg and Salmons, 1981). All stereological parameters are expressed per unit volume of myofibrillar fibre core corrected for mitochondria.

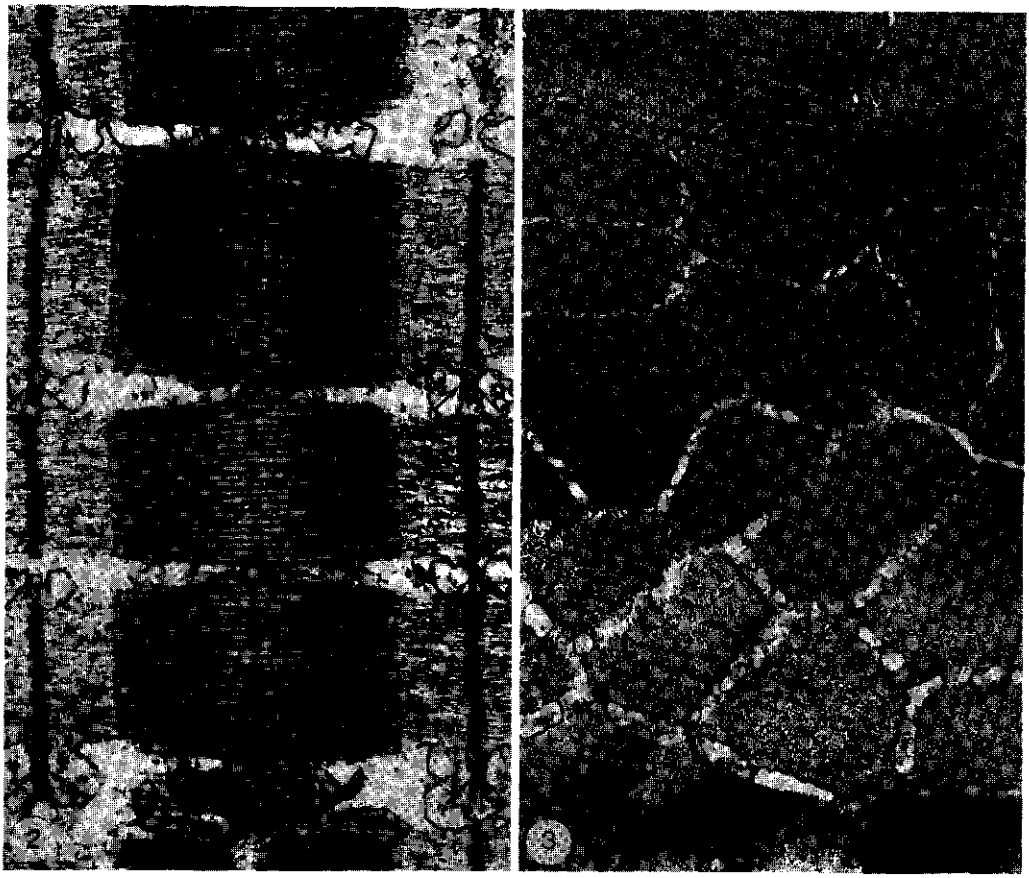

Fig. 2. T-SR contacts (arrows) in a white fibre of the LOPA (longitudinal section, 28000 x). Each triad was counted as two contacts, diads were counted as one contact; asterisk: overlap of SR and myofibrils, causing; overestimation of the SR measured in longitudinal sections.

Fig. 3. Pink fibre of the LOPA (cross sections 28000 x). Trajects of I-, A- and H-band, used for measurements of the SR, are indicated.

Table 1. Comparison of the measurement of the relative surface areas of the SR in cross sections and in longitudinal sections, mean ± sem.

		longitudinal section (point counting)				cross sections (data-tablet)
		terminal cisternae	longitudinal tubules	total SR	total SR corrected[+]	total SR
fibre types	n	μm^{-1}	μm^{-1}	μm^{-1}	μm^{-1}	μm^{-1}
pink	5	0.54 ± 0.06	2.9 ± 0.3	3.4	2.8	2.7 ± 0.7
red	5	0.28 ± 0.05	2.7 ± 0.2	3.0	2.5	2.7 ± 0.7
deep red	5	0.12 ± 0.02	2.4 ± 0.2	2.5	2.0	1.9 ± 0.5

[+] correction factor 0.8 for longitudinal tubules applied.

Stereological measurement of the SR in longitudinal sections may result in overestimation of the longitudinal tubules, due to the fact that the section thickness is greater than the tubule diameter (Weibel and Bolender, 1973, see also fig. 2). To obtain the magnitude of this error, measurements of the surface area of the SR in longitudinal- and in cross sections were compared for three types of muscle fibres. For this comparison five fibres per type from the material described by Akster (1981) were used. Of each fibre, longitudinal sections as well as cross sections were available. In micrographs of the longitudinal sections, the SR was studied as described above. In micrographs of cross sections, the SR tubules were traced and the sums of their circumferences per unit length of intermyofibrillar space were measured with a datatablet (Summagraphics Supergrid). This was done for the I band, the A band and the H band (fig. 3). As the length of the intermyofibrillar space per volume unit myofibrillar core and the dimensions of the I-, A- and H band are known (Akster, 1981), the relative surface area of the SR could be calculated. The values obtained from longitudinal sections appeared to be higher than those obtained from cross sections (table 1). Application of a correction factor of 0.8 to the surface areas of the longitudinal tubules, measured in longitudinal sections, resulted in surface areas of the total SR, similar to those measured in cross sections (table 1). These data are comparable to the 2.4 μm^{-1} found by Nag (1972) in white fibres of the axial muscles of *Salmo gairdneri* and the 2.5 μm^{-1} measured by Penney and Goldspink (1981) in oblique sections of white fibres of the axial muscles of *Carassius auratus*. The relative volumes and surface areas of the longitudinal tubules, presented in table 2, were obtained from measurements in longitudinal sections, corrected with a factor of 0.8. This has no influence on the relative differences between the fibre types.

Physiological measurements

For experiments with the fibres of the HH and the deep red fibres of the LOPA, small bundles, consisting of 5-25 muscle fibres, were used of at least five fishes. Twitches of single white fibres of the LOPA were obtained from three preparations, each from a different fish. Tetani of this fibre type were obtained from two preparations. Although this is a rather small sample, the paucity of data on the contractile properties of muscle fibres of fishes justifies the inclusion of these data in the present study.
During dissection as well as during experimentation, the muscle fibres were

continually superfused with a saline solution containing: 102 mM NaCl, 3 mM KCl, 1.25 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 0.5 mM Na_2SO_4, 2.0 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose (modified after Houston et al., 1970). The saline was oxygenated with a mixture of 95% O_2 and 5% CO_2; pH 7.4. The temperature of the saline was kept at 20°C by passing it through a heat exchanger before it entered the experimental chamber.

The preparation was attached, via the bones on which it inserted, to the small hooks of a force transducer and of a servomotor system (Granzier et al., in preparation). Measurement of twitch force was performed at fibre lengths giving optimal tetanic tension. Force was measured with a capacitive force transducer, connected to a reactance converter (Disa 51E01), with a sensitivity of 0.7 V/mN, a drift of 0.35 mN/h, a resonant frequency of 490 Hz and a compliance of appr. 5 µm/mN. The transducer was damped with silicon fluid (Dow Corning 20 cst fluid). Muscle length and -force were recorded on a chart recorder (Brush 2000) or on a dual beam storage oscilloscope (Tektronix 5103).

Stimulation of the, multiply innervated, fibres was achieved by passing current between two platinum electrodes parallel to the fibre axis. Alternating rectangular constant current pulses, 25% above threshold, were used.

RESULTS

Ultrastructure

Differences in the relative volume and surface area of the SR (tables 2c and 2f) were mainly due to differences in the relative volume and surface area of the terminal cisternae (tables 2a and 2d). No differences were found in the longitudinal tubules (tables 2b and 2e).

White fibres of the LOPA had a more extensive T-SR contact than fibres of the HH. In the deep red fibres of the LOPA, this contact was less extensive than in both other types (table 2g).

Fibres of the HH of one of the fishes appeared to have more intermyofibrillar mitochondria than those from the other four fishes. In addition, a striking difference in the length of T-SR contact was found. The extent of T-SR contact of the mitochondria-rich fibres (0.30 $\pm$ 0.06, n = 3) was similar to that of the deep red LOPA fibres. The extent of T-SR contact of the other HH fibres (0.73 $\pm$ 0.05, n = 12) was similar to that of pink fibres of the LOPA (0.69 $\pm$ 0.06, n = 15) and of pink fibres of the axial muscles (0.79 $\pm$ 0.06, n = 9). White fibres of the LOPA have a less extensive T-SR contact than white fibres of the axial muscles (0.92 $\pm$ 0.05, n = 9). The data of pink fibres and of white fibres of the axial muscles are recalculated from results described by Akster (1981).

Table 2. Relative volumes (a, b, c) and surface areas (d, e, f) of the SR and the length of T-SR contact (g), mean ± sem.

		relative volumes			relative surface areas			
		a	b	c	d	e	f	g
Fibre type	n	terminal cisternae vol%	longitudinal tubules vol%	total SR vol%	terminal cisternae μm^{-1}	longitudinal tubules μm^{-1}	total SR μm^{-1}	length of T-SR contact μm^{-2}
LOPA white	15+	2.1 ± 0.2	3.4 ± 0.4	5.5 ± 0.4	0.52 ± 0.06	2.3 ± 0.2	2.8 ± 0.2	0.80 ± 0.06
HH pink	15	1.6 ± 0.2	3.2 ± 0.3	4.8 ± 0.3	0.47 ± 0.05	2.2 ± 0.2	2.7 ± 0.2	0.65 ± 0.06
LOPA deep red	15	0.4 ± 0.1	3.5 ± 0.3	3.9 ± 0.3	0.17 ± 0.04	2.7 ± 0.2	2.9 ± 0.2	0.28 ± 0.03
LOPA deep red	15+	0.4 ± 0.2	3.5 ± 0.2	3.9 ± 0.2	-	-	-	0.33 ± 0.03

+ data from the material described by Akster, 1981.

Physiological measurements

Fibres of the three investigated fibre types reacted to a single stimulus with a twitch. This reaction could be prevented by adding 10^{-7} mM d-tubocurarine cloride to the bathing fluid, which indicates that excitation was effected by means of the nerve terminals.

Twitches of white fibres of the LOPA had a shorter time to peak tension and a shorter half relaxation time than those of fibre bundles of the HH. Twitches of deep red fibre bundles of the LOPA had a much longer time to peak tension and half relaxation time than those of the other types (fig. 4, table 3).

Table 3. Time to peak tensions and half relaxation time of isometric twitches. mean ± sem.

fibre type	n	time to peak tension msec	half relaxation time msec
white LOPA	3	14 ± 3	34 ± 2
pink HH	7	29 ± 2	46 ± 5
deep red LOPA	5	92 ± 6	151 ± 18

Fibre preparations of the HH reached maximal tetani, with a tension of 17.0 ± 0.9 N/cm^2 (mean ± sem, n = 6), at a stimulation frequency of 100-125 Hz. Maximal tetani of deep red fibre bundles of the LOPA, with a tension of 16.0 ± 1.3 N/cm^2 (n = 13), were reached at 300 - 350 Hz.
The two white fibre preparations of the LOPA reached maximal tetani, with a tension of 19 ± 2 N/cm^2, at 75 - 100 Hz.
Sag, decline of tension during a tetanus, as described by Burke et al. (1973) for muscle fibres of mammals and by Granzier et al. (1983) for fish muscle, was not apparent in the preparations of the HH and the deep red LOPA (figs. 4e and 4f). The tetani of the white fibres showed a small sag (fig. 4d). Twitches of deep red fibres reached only a low tension. Their ratio of twitch to tetanic tension was 0.11. For white fibres of the LOPA and for fibre bundles of the HH these ratios were 0.45 and 0.62, respectively.

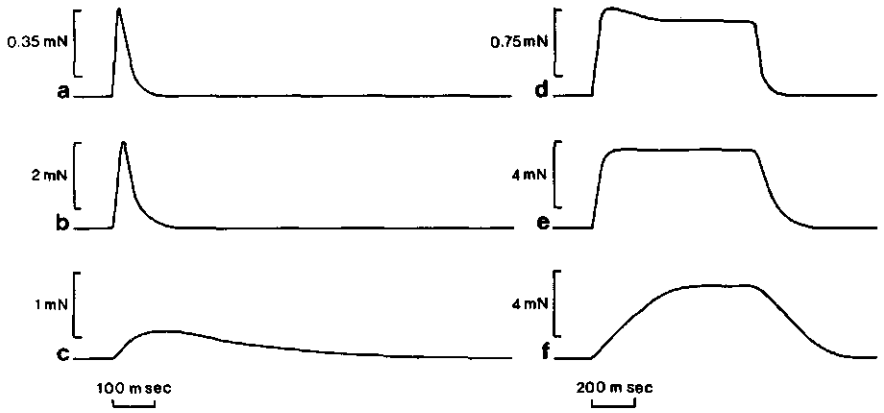

Fig. 4. Time course of isometric twitches (a, b, c) and maximal tetani (d, e, f).
a.d.: LOPA single white fibres.
b.e.: HH pink fibre bundles.
c.f.: LOPA deep red fibre bundles.

DISCUSSION

Relation between ultrastructural and physiological results

The time to peak tension of isometric twitches increased in the order: white fibres of the LOPA, fibres of the HH, deep red fibres of the LOPA. The T-SR contact and the volume and area of the terminal cisternae decreased in the same order. The T-SR contact and the terminal cisternae are both structures that are involved in the excitation-contraction coupling (Winegrad, 1970; Franzini Armstrong, 1973). An inverse relation between the twitch contraction time and the volume of the terminal cisternae was also described by Kugelberg and Thornell (1983) for fibres belonging to different rat motor units. In muscle fibre types of *Cyprinus carpio* L., however, the isometric twitch contraction time appeared to have no relation with the volume of the terminal cisternae, but only with the relative length of T-SR contact (Akster, in preparation). The relation between the twitch contraction time and the relative length of T-SR contact is similar for muscle fibre bundles of perch and carp (fig. 5). As the transmission of excitation from the T system to the SR is most probably effected at the T-SR junction (Franzini Armstrong, 1973; Eisenberg and Eisenberg,

1982), the extent of T-SR contact may determine the rate of release of calcium and thereby the rate of force development. Thus it appears that the extent of the T-SR contact is a useful ultrastructural parameter to distinguish muscle fibre types with different contractile properties.

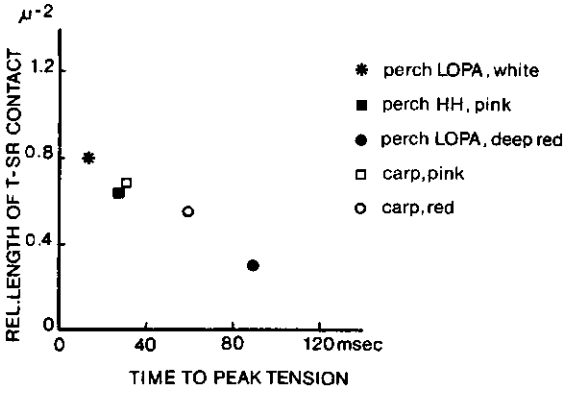

Fig. 5. Relative length of T-SR contact and time to peak tensions of the isometric twitch of different fibre types of perch and carp.

The surface of the whole SR is held responsible for the re-uptake of calcium from the sarcoplasm, which brings about the relaxation of the muscle fibre (Ebashi and Endo, 1968; Winegrad, 1970). The large differences in half relaxation time between the fibre types (table 3), however, are not accompanied by a corresponding difference in the extent of the SR; they are probably based on biochemical differences between the fibre types. Parvalbumin, a protein that acts as a calcium shuttle between the myofibrils and the SR (Gerday and Gillis, 1978; Gillis et al., 1982), is present in higher concentrations in fast than in slow muscle fibres of fishes, including those of the perch (Hamoir et al., 1981; Focant et al., 1981). Moreover, the Ca -ATPase activity of fragmented SR membranes is reported to be higher in fast than in slow muscle fibres of fishes (McArdle and Johnston, 1981).
Our data are in contrast to those of Nag (1972), who found different relative surface areas of the SR in red and white fibres of the axial muscles of *Salmo gairdneri*. Penney and Goldspink (1981) found that white fibres of the axial

muscles of *Carrassius auratus*, which were adapted to different temperatures, differed in relative area of the SR and not in the rate of calcium uptake by isolated SR fragments.
It is apparent that in the perch the differences in relaxation velocity between muscle fibre types must be due to a difference in biochemical properties of the systems that eliminate calcium and not to a difference in surface area of the SR.

A substantial contribution of the mitochondria to the calcium uptake during relaxation, as suggested by Harrison and Miller (1984) for cardiac muscle, would only enhance the discrepancy between the difference in rate of relaxation and the similarity in surface area of the SR. Such a contribution is probably relatively small because even slow skeletal muscle fibres of fish contain vast amounts of parvalbumin compared to cardiac muscle (Hamoir et al., 1981; Gerday, 1982) and because the relative volume of mitochondria that are situated between the myofibrils in slow muscle fibres of the perch is only six percent. It is likely that the subsarcolemmal mitochondria are even of less importance in calcium uptake during relaxation.

Time to peak tension of fibre types

Although descriptions of isometric twitches of fish muscle fibre types, obtained at the same temperature, are scarce, some comparisons are possible. The time to peak tension of the twitch of the white fibres of the LOPA (14 msec) is similar to that of white fibres of a dorsal muscle of the squirrelfish (12 msec, Gainer et al., 1965), while the time to peak tension of the twitch of the HH fibres of the perch (29 msec) resembles that of the pink, or intermediate, fibres of the m.hyohyoideus of the carp (32 msec, Granzier et al., 1983). Since the fibres of the HH of the perch also have a relative length of T-SR contact similar to that of known pink fibres of other muscles of the perch,it is very likely that they are pink fibres, possibly mixed with an occasional red fibre. On the basis of biochemical differences in myosin ATPase activity between white and pink fibres (Johnston et al., 1977), the twitch of white fibres was already expected to have a shorter time course than the twitch of pink fibres.

The difference in time to peak tension between red fibre bundles of the carp (59 msec, Granzier et al., 1983) and deep red fibres of the perch (92 msec) is accompanied by a difference in fibre type composition. In perch muscle as well as in carp muscle, two types of red fibres were distinguished on the basis of histochemical, electromyographical and ultrastructural data (Akster and Osse, 1978; Akster, 1981, 1983). These data indicate that in the perch the deep red

fibres are the slower of both red types. The twitches of red fibres of the carp were obtained from fibre bundles containing a mixture of both types. The bundles of deep red fibres of the perch contained only this type, which indeed has a very long time course.

Maximal tension

The tension of maximal tetani of small bundles of muscle fibres is calculated from the measured force and from the cross sectional area of the bundle, obtained by measuring the diameter. This measuring of the cross sectional area is rather inaccurate. Moreover, the influence of the connective tissue between the fibres is neglected. In skinned fibres, the cross sectional area is influenced by the effects of swelling and of the loss of soluble fibre components. Nevertheless, the tensions obtained in this study for maximal tetani of pink fibres of the HH and white fibres of the LOPA are surprisingly similar to those found by Altringham and Johnston (1982) for skinned white fibres of the axial muscles of cod, *Gadus morhua*, and dogfish, *Scyliorhinus canicula*. The maximal tension found for fast fibres of the carp (12 N/cm^2, Granzier et al., 1983) is lower, but it is higher than the values obtained by Johnston (1982) for intact fibres of cod, dogfish and of *Tilapia mossambica*.

Maximal tensions of about 18 N/cm^2 are slightly lower than those of muscle fibres of amphibia (20 - 35 N/cm^2, ter Keurs et al., 1978; Edman and Reggiani, 1984) and of mammals (24 N/cm^2, ter Keurs et al., 1984).

Red muscle fibres of perch (this study) and carp (Granzier et al., 1983) generate maximal tensions that are comparable to those developed by bundles of fast fibres of the same species. This is unexpected since red fibres have only two-thirds of the relative volume of myofibrils of fast fibres. In addition, bundles of red fibres contain more connective tissue than bundles of fast fibres (Akster, 1981, 1983). Our data contrast to those of Altringham and Johnston (1982), who found in skinned red fibres of cod and dogfish less than half the maximal tension of fast fibres. Although the limited number of observations precludes firm conclusions, our results suggest that the force development per unit cross sectional area of myofibrillar material in red fibres is at least of the same order of magnitude as it is in fast fibres and possibly larger.

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VII

FORCE-SARCOMERE LENGTH RELATIONS VARY WITH THIN FILAMENT LENGTH IN MUSCLE FIBRES OF THE PERCH (PERCA FLUVIATILIS L.)

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Isometric force (F)-sarcomere length (SL) relations were studied in eight bundles of fast twitch fibres from the hyohyoid muscle (HH) and in seven bundles of slow twitch fibres from the levator operculi anterior (LOP). Bundles of 3-20 fibres were bathed in oxygenated physiological saline; pH 7.4, 20°C. Mean active SL and steady F were measured during maximal tetani. Filament lengths in these fibres were determined by electron microscopy and corrected for shrinkage using troponin periodicity.

The F-SL relations (Fig. 1) showed a plateau at 2.00-2.20 µm (HH) and 2.50-2.70 µm (LOP); their descending limbs were linear with zero F at 3.67 µm (HH) and 4.22 µm (LOP).

In LOP muscles thick filament length was 1.40 ± 0.07 µm (± S.D., n = 15) and in HH muscles 1.31 ± 0.06 µm, the bare zone was 0.14 ± 0.01 µm and the Z-line was 0.13 ± 0.01 µm wide. Thin filament length was 0.85 ± 0.04 µm in HH and 1.19 ± 0.11 µm in LOP.

The shift of the descending limbs of the F-SL relations in these muscles is slightly less than what is expected on the basis of the differences in actin length in a sliding-filament/cross-bridge model.

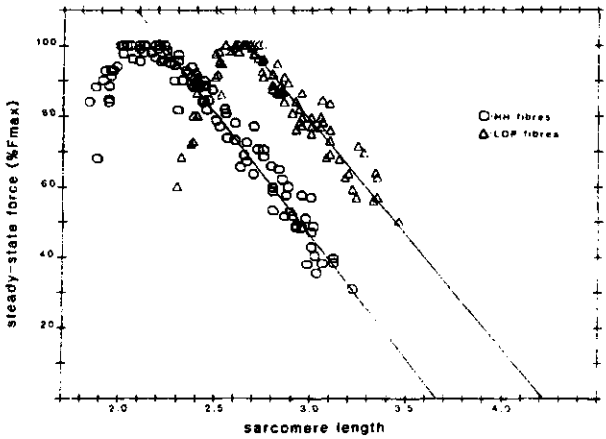

Fig. 1. The force-sarcomere length relations in muscle fibres with short (HH) and long actin in perch.

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SUMMARY

Muscles of fishes are active in a variety of movements that differ in velocity, duration and excursion length. To investigate how muscles meet the, often conflicting, demands imposed upon them by these movements, the fibre type composition of several muscles was determined. The ultrastructural and contractile properties of some of the obtained fibre types were measured and compared with their functional activity.

In head muscles as well as in body muscles of perch and carp, four types of muscle fibres were distinguished on the basis of reactions with antisera and on the pH stability of myosin ATPase. In both fishes, two fast types (white and pink) and two slow types, (red a and red b in the carp and red and deep red in the perch) were found (chapter I and chapter III). In the perch both slow types could be divided into subtypes on the basis of the activity of enzymes of the aerobic and anaerobic metabolism (chapter I).

In the perch, a muscle part consisting of red fibres, showed electromyographic activity during slow, continually repeated movements (respiration). Another part of the same muscle, containing white and pink fibres, only showed activity during fast vigorous movements (cough, suction feeding on prey) (chapter I).

To investigate the morphological basis for functional differences between the fibre types, quantitative ultrastructural studies were made. In both fishes, the four fibre types differed in the extent of contact between the T system and the sarcoplasmic reticulum (T-SR contact). White fibres had the most extensive T-SR contact, red b fibres (in the carp) and deep red fibres (in the perch) had the least extensive T-SR contact (chapter II and chapter V).

Slow as well as fast muscle fibres of perch and carp are multiply innervated. Slow fibres of the carp have a higher density of nerve terminations than fast fibres (chapter III).

The contractile properties of small bundles of pink- and red muscle fibres of the carp were measured in a nerve-muscle preparation (chapter IV). Contractile properties of white-, pink- and deep red fibres of the perch were obtained from small fibre bundles stimulated by means of long electrodes, parallel to the fibre axis (chapter VI). All investigated types reacted to a single stimulus with a twitch. Time to peak tensions increased in the order: white fibres (perch), pink fibres (perch and carp), red fibres (a mixture of the types red a and b of the carp) and deep red fibres (perch). The extent of contact between

the T system and the sarcoplasmic reticulum decreased in the same order (chapter VI, fig. 5).

The position of the T system (at the level of the Z line or at the junction between A band and I band) was not related to the extent of contact between the T system and the sarcoplasmic reticulum,as was expected from earlier qualitative studies. In muscle fibres of the perch, this position appeared to be related to the length of the actin filaments. Differences in sarcomere length- tension curves, predicted on the basis of differences in actin filament length in a sliding filament- cross bridge model (chapter II), were confirmed by experimental results (chapter VII).

Fast- and slow fibre bundles have similar maximal tetanic tensions per unit cross sectional area, although slow fibres have a lower relative volume of myofibrils (chapter IV and VI).

Red fibres of the carp have a beter endurance than pink fibres, as was measured by repeated tetanisation (chapter IV). This is in agreement with their higher relative volume of mitochondria and amount of glycogen (chapter V).

The results from this study show that fibre types present in the same muscle may differ in contraction velocity, sarcomere length- tension curve and endurance. This indicates that each fibre type is suitable to a different activity, as is indeed confirmed by their different electromyographic activity.

The obtained knowledge of the histochemical and structural characteristics of different fibre types and their functional meaning will provide a useful tool for further investigation, especially of development and growth of fish muscle.

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CURRICULUM VITAE

Hendrica Alide (Rie) Akster werd op 25 juli 1941 geboren te Oldebroek. Ze bezocht scholen te Oldebroek, Tiendeveen (gemeente Beilen), Noordseschut (gemeente Hoogeveen), Hoogeveen en Winterswijk. In 1959, na het behalen van het diploma HBS-B, trad ze als aspirant leerling laborante in dienst van het Onze Lieve Vrouwe Gasthuis te Amsterdam. In 1962 behaalde ze het diploma klinisch chemisch analiste. In die functie bleef ze tot 1966 in dienst van deze instelling.

Van 1966 tot 1970 was ze, als analiste, in dienst bij het Zoölogisch Laboratorium van de Universiteit van Amsterdam, waar ze onder leiding van Drs. W.A. Smit electronenmicroscopisch onderzoek deed aan insectespieren. Gedurende die tijd volgde ze de avondcursus MO Biologie aan de Stichting Nutsseminarium voor Pedagogiek van de Universiteit van Amsterdam en in 1969 liet ze zich ook inschrijven als studente Biologie aan deze universiteit. Van 1971 tot 1974 was ze als student-assistente in dienst van het Laboratorium voor Dierfysiologie. In 1973 deed ze kandidaatsexamen (cum laude) en in 1974 doctoraalexamen (cum laude) met als hoofdvak Algemene Dierkunde en als bijvakken Dierfysiologie en Plantenfysiologie.

Sinds 1974 is ze als wetenschappelijk medewerker in dienst van de Vakgroep Experimentele Diermorfologie en Celbiologie van de Landbouwhogeschool. Als zodanig nam ze deel aan de onderwijs- en organisatie taken van deze vakgroep en van de studierichting Biologie en bewerkte ze het in dit proefschrift beschreven onderzoek.