UNIVERSITAT DE BARCELONA

Capilarización y tipos de fibras en la musculatura esquelética de aves

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Tesis Doctoral

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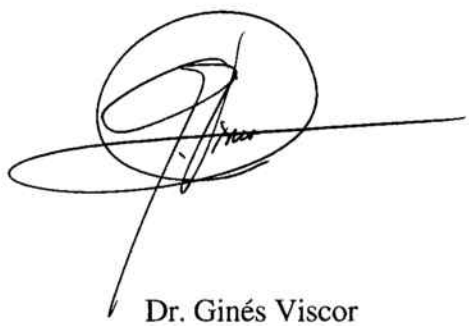
La presente Tesis Doctoral ha sido realizada por **Joan Ramon Torrella Guio** bajo la dirección del **Dr. Ginés Viscor Carrasco**. Está enmarcada dentro del programa de doctorado de Fisiología aprobado en el bienio 1990-92 y se presenta como memoria para optar al título de Doctor en Ciencias Biológicas.



Dr. Ginés Viscor
Profesor titular
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Als meus pares,
gràcies a ells tot
ha estat possible

A la Montse,
pel seu recolzament i
constant presència

Més enllà de neguits i entusiasmes,
potser el guany dels viatges és l'espai
que hi descobrim de nosaltres mateixos,
i que tal volta hauria restat fosc
sense la llum d'aquelles noves rutes.

Miquel Martí i Pol
Els Bells Camins (1988)

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Quisiera terminar este apartado citando unas palabras que el Dr. Planas pronunció en Enero de 1993, en el acto de recepción como miembro de la *Real Academia de Ciencias y Artes de Barcelona*, entre las cuales podría enmarcarse la presente tesis:

> *La morfología y la fisiología, -la estructura y la función- constituyen dos aspectos inseparables del ser vivo, y no tiene ningún sentido hablar de importancia o supremacía de una parte sobre otra, pues la vida del organismo no se entiende sin ambas.*
>
> Prof. Dr. J. Planas, 1993
> Algunos aspectos del enfoque
> fisiológico de la adaptación biológica.
> *Memorias de la Real Academia de Artes*
> *y Ciencias de Barcelona.*
> N° **908**, Vol. 52(1), p.5.

LISTA DE ARTÍCULOS

Esta tesis está basada en los siguientes artículos originales, los cuales serán referidos en el texto con la correspondiente numeración romana:

I

Fouces V, Torrella JR, Palomeque J, Viscor G (1993) A histochemical ATPase method for the demonstration of the muscle capillary network. *Journal of Histochemistry and Cytochemistry* 41:283-289.

II

Torrella JR, Fouces V, Palomeque J, Viscor G (1993) A myosin ATPase and acetylcholinesterase combined histochemical method for the demonstration of fiber types and their innervation pattern in skeletal muscle. *Histochemistry* 99:369-272.

III

Torrella JR, Fouces V, Palomeque J, Viscor G (1993) Innervation distribution pattern, nerve ending structure and fiber types in pigeon skeletal muscle. *Anatomical Record* 237:178-186.

IV

Torrella JR, Fouces V, Palomeque J, Viscor G (1996) Capillarity and fibre types in locomotory muscles from wild mallard ducks. *Journal of Comparative Physiology B* 166:164-177.

V

Torrella JR, Fouces V, Palomeque J, Viscor G (1997) Capillarity and fibre types in locomotory muscles of wild common coots (*Fulica atra*). En manuscrito enviado al *Journal of Morphology*.

VI

Torrella JR, Fouces V, Palomeque J, Viscor G (1997) Capillarity and fibre types in locomotory muscles from wild yellow-legged gulls (*Larus cachinnans*). En manuscrito enviado al *Physiological Zoology*.

VII

Torrella JR, Fouces V, Palomeque J, Viscor G (1997) Comparative skeletal muscle fibre morphometry among wild birds with different locomotory habits. En manuscrito enviado al *Journal of Anatomy*.

ABREVIATURAS UTILIZADAS

AChE	acetilcolinesterasa
ALD	músculo anterior latissimus dorsi
BRC	músculo brachialis
CCA	número de capilares por cada 1.000 μm^2 de área de fibra (*capillary counts per area*)
CCP	número de capilares por cada 100 µm de perímetro de fibra (*capillary counts per perimeter*)
CD	densidad capilar (*capillary density)*
C/F	cociente entre el número de capilares y el número de fibras (*capillary-to-fibre ratio*)
EDL	músculo extensor digitorum longus
EMR	músculo extensor metacarpi radialis
FCSA	área de la sección transversal de la fibra (*fibre cross-sectional area*)
FD	densidad de fibras (*fibre density*)
FG	*fast glycolytic*
FOG	*fast oxidative glycolytic*
FPER	perímetro de la fibra (*fibre perimeter*)
GLE	músculo gastrocnemius lateralis (pars externa)
α-GPDH	α-glicerofosfato deshidrogenasa
ITC	músculo iliotibialis cranialis
mATPasa	miosina adenosín-trifosfatasa
MDD	distancia de difusión máxima (*maximal diffusion distance*)
NCF	número de capilares por fibra
%OFA	porcentaje en área de fibras oxidativas
%OFN	porcentaje en número de fibras oxidativas
PEC	músculo pectoralis
PRN	músculo pronator superficialis
SCH	músculo scapulohumeralis caudalis
SDH	succinato deshidrogenasa
SMP	músculo serratus metapatagialis
SO	*slow oxidative*
SOL	músculo soleus
SW	*slow white*
TSC	músculo triceps scapularis o scapulotriceps

ÍNDICE

Presentación y Objetivos

The most characteristic features of birds are their possession of feathered wings and their ability to fly. It was this unique combination that bestowed upon ancient birds not only the ability to conquer the air but also the right of survival over the ruling reptiles of the Mesozoic age and amidst the rising mammals. In the process of transformation from clumsy reptilian body into the compact and elegant flying machine, there were several fundamental changes in the structure and physiology of the various organ systems. Among them, those embracing the musculoskeletal system are the most striking.

John C. George and Andrew J. Berger, 1966

Esta tesis consiste en una serie de artículos que muestran los resultados del trabajo realizado entre 1991 y 1996 en el laboratorio de Fisiología (Facultad de Biología) de la Universidad de Barcelona. Utilizando métodos histoquímicos, se han investigado diferentes aspectos referentes a la capilarización, composición y características de las fibras de la musculatura esquelética en cuatro especies de aves.

Siete artículos escritos en inglés integran esta tesis. Cuatro de ellos (**I-IV**) ya han sido publicados y los otros tres (**V-VII**) están actualmente (Abril de 1997) sometidos a consideración editorial para su publicación en revistas especializadas. Se adjunta una copia de los cuatro primeros artículos en el formato original de la revista donde han aparecido, mientras que los tres últimos artículos se muestran en un formato de texto estándar común. Precede a todos ellos una sección dividida en los apartados habituales de las publicaciones científicas: Introducción, Material y Métodos, Resultados, Discusión, Conclusiones y Referencias. Esta sección resume y justifica la unidad temática de todo el trabajo, desglosado en los artículos posteriores, y muestra una aproximación comparada de los resultados que no siempre ha sido posible presentar en dichos artículos. Para ello, y aún a riesgo de generar algunas repeticiones, se ha dotado a esta sección de una cierta extensión. No obstante, es importante destacar que el grueso de esta tesis lo constituyen los siete artículos mencionados, ya que en ellos se discuten profusamente todos los resultados obtenidos y se comparan con la literatura científica existente, que no es escasa, sobre la histoquímica y la fisiología de la musculatura esquelética en aves.

Los artículos **I** y **II** integran la primera parte, claramente metodológica, de la investigación. Consisten en dos técnicas relevantes de tinción histoquímica. La primera permite cuantificar el aporte capilar al músculo en cortes transversales (**I**) y la segunda pone de manifiesto, sobre un mismo corte de tejido longitudinal o transversal, las características de inervación y el tipo de fibra muscular según su actividad mATPasa (**II**). El artículo **III** es un estudio monográfico sobre los tipos de fibras de la paloma (*Columba livia*) en diversos músculos locomotores y posturales. Los artículos **IV**, **V** y **VI** son también tres monografías sobre la capilarización y los tipos de fibras de seis músculos de aves. En ellos se estudia la musculatura motora implicada en el vuelo, la natación y la locomoción terrestre de tres especies de aves salvajes con hábitos locomotores distintos: el ánade real (*Anas platyrhynchos*), la focha común (*Fulica atra*) y la gaviota patiamarilla (*Larus cachinnans*). Cierra la investigación el artículo **VII**, donde se estudian conjuntamente las características morfométricas de las fibras musculares de estas tres especies.

Los principales objetivos de esta investigación han sido:

1. Diseñar una metodología histoquímica que permita un análisis cuantitativo de la red capilar del músculo esquelético y un análisis cualitativo del tipo de fibra muscular y su inervación.

2. Estudiar las diferencias cualitativas y cuantitativas en los patrones de inervación de los distintos tipos de fibras presentes en la musculatura esquelética de aves, tomando a la paloma como modelo de referencia.

3. Estudiar las diferencias a nivel histoquímico, morfométrico y de abastecimiento capilar entre los distintos tipos de fibras presentes en la musculatura de aves.

4. Describir las variaciones regionales en seis músculos locomotores de tres especies de aves (ánade real, focha común y gaviota patiamarilla) a nivel de las variables mencionadas.

5. Analizar la capilarización, la composición fibrilar y la morfometría de la musculatura motora de las tres especies de aves salvajes mencionadas y ver hasta qué punto estas variables están adecuadas a los hábitos locomotores de cada especie, constatando las diferencias específicas que se presentan en cada caso.

Introducción

1. Anatomía fisiológica del músculo esquelético

1.1. Estructura macroscópica

La producción de fuerza es la función principal de la musculatura esquelética de cualquier animal. Dicha función es llevada a cabo, de manera controlada, por un tipo de célula altamente especializado que forma la unidad básica de los músculos esqueléticos de los vertebrados. Desde la primera descripción en varias especies de la naturaleza fibrilar de la musculatura esquelética (Bowman 1840), la célula muscular recibe el nombre de **fibra muscular**. Estas células poseen una estructura cilíndrica, son multinucleadas y se disponen en paquetes o fascículos rodeados de una capa de tejido conectivo. El conjunto de todos los fascículos constituye el músculo entero. Entre las fibras musculares de cada fascículo se encuentran las estructuras responsables del abastecimiento metabólico y control nervioso del tejido muscular: los capilares sanguíneos y los terminales axónicos de los nervios motores. En los extremos de los músculos, las membranas plasmáticas de las fibras musculares se fusionan con fibras tendinosas que, al agruparse en haces, constituyen los tendones. Mediante éstos, los músculos generalmente se insertan en los huesos formándose un sistema de palancas que facilita no sólo la transmisión de fuerza, sino también el almacenamiento de energía elástica que, en muchos casos, es reutilizada para reducir los costes de la locomoción (Alexander 1988).

1.2. La fibra muscular

Cada fibra muscular se compone de unidades repetidas, consistentes en pequeños fascículos cilíndricos que contienen el material responsable de la contracción muscular. Estos pequeños fascículos, que reciben el nombre de **miofibrillas**, están formados básicamente por dos tipos de proteínas organizadas en forma de filamentos: la **actina** y la **miosina**. Los filamentos gruesos de miosina y los filamentos finos de actina se disponen intercalados en estructuras alineadas que reciben el nombre de **sarcómeros**, los cuales quedan unidos por medio de una estructura especializada denominada **disco Z**. La repetición en serie de los sarcómeros, a lo largo del eje longitudinal de la fibra muscular, confiere al tejido muscular esquelético su típica apariencia bandeada bajo el microscopio (véanse, por ejemplo, las excelentes microfotografías de los estudios sobre el pectoral de paloma de Ashhurst 1969 y Grinyer & George 1969).

1.2.1. Filamentos de miosina

Los filamentos de miosina son polímeros de esta proteína. Cada molécula de miosina está constituida por seis cadenas polipeptídicas: dos cadenas pesadas (**MHC**, *myosin heavy chain*) y cuatro cadenas ligeras (**MLC**, *myosin light chain*). Las porciones carboxi-terminales de las MHC se entrelazan entre sí formando una espiral doble (la cola de miosina), mientras que las porciones amino-terminales se separan formando dos dominios globulares (las cabezas de miosina). En el lugar de unión de la cabeza y la cola de miosina se disponen las MLC, dos en cada dominio globular de la MHC (véase la revisión de Schiaffino & Reggiani 1994). Un pequeño segmento de la porción espiral de cada molécula

de miosina se extiende hacia un lado, junto con el dominio globular de su cabeza, dando lugar a un brazo que se proyecta desde el filamento (H.E. Huxley 1963). Los brazos y las cabezas de miosina se denominan globalmente **puentes cruzados** y juegan un papel importante en el proceso de la contracción muscular. Desde el trabajo de Engelhardt & Ljubimova (1939) se conoce la actividad ATPasa de la miosina que, al interactuar con los filamentos de actina y formar el sistema actomiosina-ATP (Szent-Györgyi 1951), aporta la energía necesaria para la contracción muscular como resultado de la hidrólisis del ATP (H.E. Huxley 1969).

1.2.2. Filamentos de actina

Los filamentos de actina están formados por tiras helicoidales de moléculas de actina globulares, que se enrollan con filamentos de otra proteína polimérica, la tropomiosina, la cual en estado de reposo cubre los lugares de interacción entre la actina y la miosina. El tercer componente del filamento de actina lo constituye un complejo de proteínas globulares de troponina que se distribuye, de manera regular, sobre los filamentos de tropomiosina. De este complejo depende la unión al Ca^{2+} (Ebashi *et al.* 1969; Holmes *et al.* 1990) que, como se verá más adelante, inicia el proceso de contracción.

1.2.3. Retículo sarcoplasmático

Además de la presencia de miofibrillas y miofilamentos, la fibra muscular presenta algunas otras peculiaridades como resultado de su elevado grado de especialización en la producción de fuerza muscular. La más destacable es la estructura de su retículo endoplasmático, que está muy desarrollado y recibe el nombre de **retículo sarcoplasmático**. Este sistema membranoso intracelular se extiende rodeando en forma de túbulos transversos (**túbulos T**) a las miofibrillas. En realidad el retículo sarcoplasmático es una prolongación de la membrana plasmática ya que los lugares donde éste se origina están abiertos al exterior, comunicando con el líquido extracelular y transfiriendo varios factores desde el medio externo al líquido intracelular (Endo 1964; H.E. Huxley 1964). A ambos lados de los túbulos T colindan unas expansiones membranosas en forma de cámaras, denominadas **cisternas**, en cuyo interior se encuentran elevadas cantidades de iones Ca^{2+}. Dos cisternas y un túbulo T constituyen una **tríada** que se comunica con las demás mediante largos túbulos longitudinales que rodean a cada miofibrilla.

1.3. Inervación de la fibra muscular

Galeno (131-201 A.D.) en el siglo II postuló que el nervio y el músculo son funcionalmente inseparables, ya que observó que la contracción del músculo sólo tenía lugar si los nervios que lo abastecían estaban intactos. Galeno creía que los nervios eran el medio a través del cual el espíritu animal fluía a los músculos. No fue hasta finales del siglo XVIII cuando Galvani (1791) demostró que el músculo puede ser estimulado eléctricamente y que el nervio es de hecho una fuente y un transmisor de energía eléctrica. Hoy sabemos que las neuronas son capaces de generar y conducir señales eléctricas y también de excitar a la célula muscular mediante substancias químicas liberadas en los terminales nerviosos (véase, por ejemplo, Matthews 1991; Vrbová *et al.* 1995).

Cada fibra muscular está inervada por **motoneuronas**, células nerviosas que nacen en las astas anteriores de la médula espinal y poseen largos axones rodeados de mielina. El punto de contacto entre las motoneuronas y la fibra muscular recibe el nombre de **unión neuromuscular** y posee algunas particularidades interesantes. El extremo del axón se ramifica en la superficie de la fibra muscular formando el terminal nervioso, que puede tener diferente morfología según las características funcionales y el tipo de fibra muscular inervado. En cualquier caso, en la parte final del axón motor se da una acumulación de **vesículas sinápticas** donde se almacena la **acetilcolina**, el neurotransmisor que servirá de señal química entre la neurona y la fibra muscular. También es posible observar una gran cantidad de mitocondrias que proporcionarán la energía necesaria para sintetizar la acetilcolina. La porción de membrana plasmática de la fibra muscular que está en contacto con la unión neuromuscular posee numerosos pliegues o hendiduras que aumentan la superficie de actuación del transmisor sináptico. En estas hendiduras también se localizan los receptores de acetilcolina que, al combinarse con este neurotransmisor, producirán la despolarización de la fibra muscular. También se encuentran en esta zona grandes cantidades del enzima **acetilcolinesterasa**, el cual es el responsable de la degradación de la acetilcolina una vez ésta ha actuado en la superficie de la fibra muscular.

Una motoneurona individual tiene muchas ramificaciones y cuando es activada produce la contracción de todas las fibras musculares que inerva. Sherrington (1906) denominó **unidad motora** a la organización formada por la motoneurona, sus axones terminales y las fibras musculares que inerva. Este autor sugirió que la unidad motora constituía la unidad funcional más pequeña de control del movimiento dentro del sistema nervioso central. Aunque con algunas excepciones (Pierobon Bormioli *et al.* 1980; Mascarello *et al.* 1983; Rossi 1990; Kaminski *et al.* 1996) la mayor parte de las fibras musculares de mamíferos presentan una **inervación focal**, es decir, las fibras musculares únicamente están inervadas por un terminal nervioso (Coërs 1967). Sin embargo en otros grupos de vertebrados puede darse además **inervación multiterminal**, si un mismo axón se ramifica para inervar una misma fibra en diferentes puntos (Coërs 1967), o **inervación polineural**, si una fibra es inervada en diferentes puntos por diferentes motoneuronas (Johnston 1981).

1.4. Mecanismo de la contracción muscular

El mecanismo de contracción muscular es un proceso complejo que requiere de varios pasos (Frischknecht *et al.* 1990):

(1) Cuando el impulso nervioso llega al terminal axónico se libera acetilcolina de las vesículas sinápticas al espacio sináptico de la unión neuromuscular. Al combinarse el neurotransmisor con los receptores de acetilcolina, el sarcolema y todo su sistema de túbulos T se despolariza, iniciándose un potencial de acción y activándose la fibra muscular (Katz 1966).

(2) Al despolarizarse la fibra muscular, se induce la liberación de Ca^{2+} de las cisternas del retículo sarcoplasmático al citoplasma celular. La presencia de un sistema de túbulos T bien desarrollado aumenta la velocidad de este proceso. Aunque la primera evidencia que demostró la importancia del Ca^{2+} en la contracción muscular es muy antigua (Ringer & Buxton 1887), así como también las primeras demostraciones del papel regulador que este ion juega en la contracción muscular (Kamada & Kinosita 1943;

Heilbrunn & Wierczinski 1947), el rol exacto de los iones Ca^{2+} en el acoplamiento de excitación-contracción de las fibras musculares todavía constituye materia de creciente debate científico (véase la reciente revisión de Melzer *et al.* 1995).

(3) Los iones de Ca^{2+} difunden al interior de las miofibrillas donde se unen a la troponina, la cual activa la interacción entre actina y miosina a través de la tropomiosina, iniciándose el proceso de contracción (Rüegg 1986; Ashley *et al.* 1991).

(4) La contracción muscular tiene lugar como resultado de la interacción entre la actina y la miosina, acortándose el sarcómero como consecuencia del deslizamiento activo entre los filamentos de ambas proteínas (A.F. Huxley & Niedergerke 1954; H.E. Huxley & Hanson 1954). Este deslizamiento es llevado a cabo por los puentes cruzados formados por las cabezas de miosina, cada uno de los cuales actúa como un generador de fuerza independiente, tirando del filamento de actina hacia el centro del sarcómero. La actividad ATPasa de la cabeza de miosina hidroliza el ATP, proporcionando la energía necesaria para generar fuerza muscular. Desde la reciente determinación de la estructura de los puentes cruzados, por cristalografía de rayos X (Rayment *et al.* 1993*a*,*b*), la comprensión de los detalles de este proceso ha sufrido un gran avance y ha sido objeto de una excelente revisión por Goldspink (1996).

(5) El proceso de relajación tiene lugar por transporte activo de los iones Ca^{2+} a sus reservas originales en las cisternas del retículo sarcoplasmático. Al disociarse el Ca^{2+} de la troponina, el músculo vuelve a su estado inicial de relajación (A.F. Huxley 1974).

2. Tipos de fibras musculares

2.1. Primeras clasificaciones

Todos los músculos esqueléticos de vertebrados poseen características generales que han sido resumidas en las secciones anteriores. A pesar de ello, se dan algunas variaciones que revelan una pronunciada heterogeneidad en la población de las fibras musculares, la cual es la base de los diferentes comportamientos fisiológicos del músculo.

En 1678 Stefano Lonrenzini describió diferencias de color en los músculos de la pata de conejo que le llevaron a distinguir entre músculos rojos y músculos blancos (Ciaccio 1898). No obstante, fue Ranvier (1874*a*) quien, con la ayuda del microscopio, describió por primera vez algunas diferencias histológicas entre ambos tipos de músculo, realizando además experiencias que le permitieron establecer algunas de sus propiedades fisiológicas: los músculos rojos se contraían y relajaban más lentamente que los músculos blancos. Los estudios histológicos de Knoll (1881) y Grützner (1883) mostraron diferencias entre fibras musculares individuales e identificaron fibras "claras" y fibras "opacas". Años más tarde Krüger (1949) propuso una clasificación basada en la estructura miofibrilar de las células musculares: fibras **Fibrillenstruktur**, si su citoplasma observado en sección transversal tenía una apariencia de "fibrillas"; y fibras **Felderstruktur**, si la apariencia era "en campos". Con la aparición de las tinciones histoquímicas estas clasificaciones quedaron en desuso para dar paso a nomenclaturas basadas en las características metabólicas de la fibra muscular.

2.2. Propiedades histoquímicas, metabólicas y fisiológicas

Un paso importante en el conocimiento de la heterogeneidad muscular fue la aplicación de la tinción histoquímica para la adenosín-trifosfatasa, ATPasa (Padykula & Herman 1955; Engel 1962), ya que posibilitó la diferenciación entre fibras lentas o de tipo I y rápidas o de tipo II. A partir de estos hallazgos aparecieron varias nomenclaturas y sistemas de clasificación de las fibras musculares (Stein & Padykula 1962; Romanul 1964; Brooke & Kaiser 1970; Guth & Samaha 1970; Ashmore & Doerr 1971), todos ellos basados en la combinación de la tinción ATPasa (pretratamientos ácidos o alcalinos) con otras técnicas histoquímicas que ponen de manifiesto enzimas oxidativos y glicolíticos (Seligman & Rutenburg 1951; Dubowitz & Pearse 1960; Wattenberg & Leong 1960). Esta gran proliferación de técnicas histoquímicas y sistemas de nomenclatura conllevó una gran confusión que roza lo incomprensible si se tiene en cuenta que no todos los sistemas de clasificación son equivalentes entre sí (Green *et al.* 1982; Dhal & Roald 1991). Afortunadamente, profundizando en las propiedades fisiológicas y las características metabólicas de las fibras musculares se han encontrado correlaciones con sistemas de clasificación histoquímicos.

Por simplicidad se han modificado los procedimientos histoquímicos hasta el punto de poder discriminar sólo tres o cuatro tipos de fibras. Los esquemas de tipificación basados en la sensibilidad de la mATPasa a diferentes pH y en los distintos perfiles metabólicos y enzimáticos se han convertido en una práctica estándar que muestra una gran correspondencia entre tipos de fibras y tipos de unidades motoras (Pette & Staron 1990; Vrbová *et al.* 1995). Utilizando la actividad de diversos enzimas a partir de determinaciones bioquímicas, Peter *et al.* (1972) clasificaron las fibras musculares de mamíferos en tres tipos básicos: **SO** (*slow oxidative*), **FOG** (*fast oxidative glycolytic*) y **FG** (*fast glycolytic*). Esta nomenclatura, pese a ser una simplificación "académica" de la realidad muscular, ha tenido una gran aceptación en el estudio de la fisiología del músculo esquelético pues atribuye, a cada tipo de fibra, unas características metabólicas que posteriormente se demostraron estar relacionadas con las propiedades fisiológicas de las diferentes unidades motoras. Burke *et al.* (1971, 1973, 1974), utilizando la técnica de depleción de glicógeno de Edström & Kugelberg (1968), identificaron de manera directa tres tipos de unidades motoras. Primero diferenciaron dos grupos de acuerdo con su velocidad de contracción: las unidades motoras de contracción lenta (**S**, *slow*) y las de contracción rápida (**F**, *fast*). En segundo lugar, las unidades motoras de contracción rápida fueron subdivididas según su comportamiento frente a la fatiga en unidades motoras resistentes a la fatiga (**FR**, *fast fatigue-resistant*) y unidades motoras de fatiga rápida (**FF**, *fast fatigable*). Los trabajos de Burke y sus colaboradores son especialmente relevantes no sólo por presentar una clasificación fisiológica de las unidades motoras, sino también porque demostraron que las unidades motoras S contienen fibras SO, las unidades motoras FR fibras FOG y las unidades FF se componen de fibras FG.

2.3. Bases moleculares de la heterogeneidad fibrilar

Las diferencias que se observan después de preincubaciones ácidas o alcalinas en las reacciones histoquímicas de la mATPasa son debidas a la presencia de diferentes isoformas de la molécula de miosina, presentando cada tipo de isomiosina sensibilidades diferentes a pH distintos (Peters 1989; Staron & Pette 1990). Por esta razón, la complejidad del

músculo esquelético como tejido sólo puede ser entendida investigando la composición del contenido individual de sus moléculas de miosina. En los últimos años se han identificado varios tipos de MHC en la musculatura de mamíferos (véase Pette & Staron 1990; Schiaffino & Reggiani 1994) y en la de aves (Bandman *et al.* 1990; Gauthier & Orfanos 1993; Reiser *et al.* 1996, Rosser *et al.* 1996), pudiéndose presentar una determinada isoforma de manera única o coexpresarse junto con otras en una misma fibra muscular (Termin *et al.* 1989; Talmadge *et al.* 1995; Rivero *et al.* 1996).

Se sabe, desde el trabajo de Bárány (1967), que la actividad de la mATPasa de la musculatura rápida es muy superior a la de la musculatura lenta, existiendo una correlación entre la fuerza desarrollada por el músculo y la actividad de la mATPasa. Estudios más recientes han demostrado que la velocidad de contracción de cada fibra muscular varía principalmente en función del contenido relativo de las isoformas de las MHC (Bottinelli *et al.* 1991; Reiser *et al.* 1985, 1996). Además de la variabilidad en las MHC, las cadenas ligeras de miosina y las proteínas reguladoras asociadas a los filamentos de actina (troponina y tropomiosina) también presentan polimorfismos moleculares que, aunque en menor medida, influyen en las diferentes velocidades de contracción de los distintos tipos de fibras (véase la revisión de Pette & Staron 1990). Esta multiplicidad de isoformas da como resultado el considerable espectro de tipos de fibras que se observa a partir de los métodos ATPasa histoquímicos (Billeter *et al.* 1981; Hämäläinen & Pette 1995). Todo ello refleja las múltiples actividades que las fibras musculares realizan con el fin de adaptarse a las diferentes demandas funcionales, creando así una respuesta ajustada a las necesidades locomotoras ambientales del organismo (Vrbová *et al.* 1995).

3. Capilarización y capacidad oxidativa del músculo

3.1. Abastecimiento sanguíneo del músculo esquelético

Las células musculares, como cualquier tipo de célula, necesitan un continuo aporte de oxígeno y nutrientes para efectuar sus funciones vitales. El tejido muscular posee un sistema microvascular bien desarrollado en forma de capilares sanguíneos con diámetros medios de 3 a 5 μm según el músculo considerado (Wiedeman 1984; Mathieu-Costello 1991). Una cantidad importante de oxígeno se descarga en menos de 1 segundo en el momento que los eritrocitos atraviesan estos capilares. La eficacia de esta descarga depende del adecuado diseño de la microvasculatura (Weibel 1984). Este autor denomina **unidad microvascular** a la unidad básica encargada del intercambio gaseoso a nivel tisular. El comienzo de la unidad lo forma una arteriola que, como parte final del árbol arterial, se encarga de regular el flujo de sangre al músculo. Se establece una conexión entre esta arteriola y los capilares por medio de cortos vasos o **precapilares**, los cuales también poseen pequeños esfínteres capaces de regular el paso de sangre a la red capilar. Los capilares forman un amasijo de interconexiones, consistente en un número variable de segmentos que se ramifican y reunifican repetidas veces, hasta formar las porciones **postcapilares** que finalmente desembocan en las vénulas. Las redes capilares de unidades vasculares vecinas están, además, conectadas por segmentos capilares, de manera que los capilares de todo el músculo forman una extensa y continua red abastecida en diversos puntos por arteriolas y drenada por vénulas (véase Weibel 1984).

3.2. Arquitectura de la red capilar

La distribución anatómica de los capilares en la musculatura esquelética fue descrita por primera vez por Ranvier (1874*b*) y Spalteholz (1888). Ambos autores mostraron que la mayoría de los capilares se orientan paralelamente a las fibras musculares, interconectándose entre ellos mediante algunos capilares dirigidos de manera transversal. Krogh (1919) inició el camino hacia la cuantificación de la red capilar del músculo estimando la **densidad capilar** de la sección transversal, es decir, contando el número de capilares por mm^2 de área transversal. Debido a la mencionada distribución longitudinal de los capilares respecto a las fibras musculares este parámetro, obtenido en secciones bidimensionales de tejido, representa una buena descripción del tamaño de la red capilar. No obstante, como que el aporte de oxígeno a las células musculares es un proceso complejo donde intervienen transportes por difusión y convección (Groebe 1992), se requieren más variables para cuantificar de manera adecuada las dimensiones de la vascularización del músculo y sus implicaciones funcionales (Egginton & Ross 1992). Así pues, los valores de la densidad capilar se suelen acompañar de otras variables, tales como las distancias intercapilares y de difusión, el número de capilares por fibra y las medidas morfométricas (área y perímetro), que describen el aporte vascular a las fibras individuales. Otro parámetro muy utilizado es el *capillary-to-fibre ratio*, el cual es relativamente independiente de las posibles variaciones en el diámetro de las fibras (Plyley & Groom 1975; Andersen & Henriksson 1977; Gray & Renkin 1978; Hudlická 1985).

La evidencia de que los capilares se distribuyen en realidad alrededor de las fibras musculares con cierto grado de tortuosidad (Andersen & Kroese 1978; Ishikawa *et al.* 1983) llevó a Mathieu *et al.* (1983) a diseñar un método para estimar la densidad capilar longitudinal (*"capillary length density"*). Esta nueva aproximación permite el cálculo de parámetros tridimensionales funcionalmente relevantes en la microvasculatura del músculo, tales como el volumen de sangre capilar disponible para el intercambio gaseoso de las fibras o el área de superficie capilar a través de la cual el oxígeno difunde a las células (Hoppeler & Kayar 1988). Al utilizarse parámetros de medida de la red capilar tridimensionales, es posible compararlos con los volúmenes mitocondriales, que también es una medida tridimensional, y evaluar si existe una correlación entre la liberación de oxígeno y el consumo mitocondrial.

Con la aparición de las técnicas de *microcorrosion cast* para el estudio de la microvasculatura tisular (Murakami 1971) se ha podido estudiar la distribución geométrica tridimensional de los capilares alrededor de las fibras musculares. La técnica consiste, a grandes rasgos, en perfundir una resina por vía aórtica y dejarla polimerizar durante un cierto tiempo. Posteriormente se tratan los músculos con KOH concentrado que disuelve completamente el tejido, permaneciendo únicamente el *cast* o molde de la red vascular, cuya morfología tridimensional se puede observar en un microscopio electrónico de barrido. Potter *et al.* (1991) aplicaron esta técnica en el pectoral de paloma y demostraron que la distribución de los capilares alrededor de las fibras posee dos patrones distintos que son consecuencia de los elevados requerimientos metabólicos de este músculo:

a) Un gran número de capilares va paralelo a la fibra muscular y presenta morfologías sinuosas que tienen como función principal asegurar la transferencia de oxígeno a la fibra muscular durante la contracción. Todo ello crea una tensión de oxígeno uniforme alrededor de la fibra.

b) Se observa una gran cantidad de capilares, dispuestos de forma perpendicular a la fibra muscular y agrupados en estructuras en forma de abanicos, que se encuentran preferentemente situados en las terminaciones venulares de la red capilar. De esta manera incrementan el área de difusión de oxígeno en aquellos lugares del tejido donde la tensión es más reducida.

Estos trabajos en el pectoral de paloma, junto con otros estudios comparados sobre la capilarización muscular (véase para revisión Mathieu-Costello 1993), demuestran que los capilares poseen distribuciones particulares que varían según las restricciones metabólicas impuestas por los diferentes niveles de actividad de cada músculo.

3.3. Variaciones en la densidad capilar

La red capilar varía de manera significativa en relación a las necesidades funcionales del músculo y viene determinada por las propiedades metabólicas de las fibras que lo componen. Los trabajos pioneros de Ranvier (1874*b*) y Krogh (1919) demostraron por primera vez que los músculos con una función más oxidativa tienen un mayor abastecimiento capilar. Estos hallazgos han sido corroborados posteriormente, encontrándose altos niveles de correlación entre variables que cuantifican la red capilar y indicadores de la actividad oxidativa del músculo (véase la revisión de Hudlická 1985). En el caso de la densidad capilar, y a título de ejemplo, cabe destacar la dependencia que este parámetro presenta con la actividad de diversos enzimas oxidativos y con los volúmenes mitocondriales de la fibra muscular (Bass *et al.* 1969; Hudlická 1982; Hoppeler *et al.* 1981). Otra característica importante de la red capilar en la musculatura esquelética es su plasticidad, es decir, la capacidad de adaptarse a cambios metabólicos inducidos por diferentes factores. Entre ellos cabe destacar el entrenamiento (en aves: Butler & Turner 1988; en mamíferos: revisiones de Henriksson 1992 y Degens & Veerkamp 1994), la hipoxia (Valdivia 1958; León-Velarde *et al.* 1993), la estimulación eléctrica del músculo (Hudlická & Price 1990; Bigard *et al.* 1993), la exposición al frío (Duchamp *et al.* 1992) y la administración crónica de diversas substancias como hormonas tiroideas (Capo & Sillau 1983) o etanol (Vila 1996).

No obstante, algunos estudios no demuestran una clara asociación entre la capacidad oxidativa del músculo y su densidad capilar (Maxwell *et al.* 1980). Además, las fibras glicolíticas poseen aparentemente mayores cantidades de capilares de los que cabría esperar en relación a su bajo contenido mitocondrial (Weibel 1984). Este hecho se interpreta como una consecuencia de la doble función que los capilares musculares desempeñan. En primer lugar, aportan y eliminan los gases respiratorios y, en segundo lugar, se encargan de suministrar substratos energéticos y de eliminar substancias metabólicas de desecho, como por ejemplo el lactato (Weibel 1984; Hudlická *et al.* 1987).

4. La locomoción en aves

La mayoría de las aves pueden desarrollar un tipo de locomoción que el hombre, sin la ayuda de la técnica, es incapaz de realizar: el vuelo. Esta envidiable capacidad siempre nos ha fascinado y ha atraído la atención de artistas y científicos de todas las épocas. Prácticamente cada aspecto de la anatomía de las aves refleja las restricciones establecidas por el vuelo, siendo la configuración de los músculos implicados en este tipo de locomoción uno de los aspectos más importantes para entender las adaptaciones ambientales de cada especie (Proctor & Linch 1993).

No obstante, a pesar de la importancia predominante del vuelo como forma de locomoción, para entender los diferentes aspectos de la biología de las aves hay que considerar también las otras formas de locomoción que estos animales desarrollan. La mayor parte de las aves poseen dos sistemas locomotores independientes: las extremidades superiores o alas y las inferiores o patas (véase Butler 1991). Además de ser utilizadas durante el vuelo, en algunas especies las alas intervienen también en la propulsión subacuática, mientras que las patas son utilizadas en la locomoción terrestre, la natación y el buceo.

4.1. Locomoción aérea

> *Li nervi e muscoli dell'uciello sanza comparazione essere di megior potenzia che quelli dell'omo, (...) che tutta la carnosità di tanti muscoli e polpe del petto essere fatti a benefizio e aumento del moto delle alie, (...) che aparechia potenzia grandissima all'uciello.*
>
> I Manoscritti di Leonardo da Vinci. Codice sul Vuolo degli Uccelli e varie altre Materie. Ed. E. Rouveyre, 1893. Paris.

Estos primeros apuntes sobre la implicación de la musculatura pectoral de las aves en el vuelo, escritos en 1505 por Leonardo da Vinci (1452-1519), están hoy fuera de toda duda. El vuelo constituye un movimiento cíclico con dos fases principales: el descenso y la elevación del ala (George & Berger 1966). Durante el descenso del ala el principal músculo que presenta actividad es el **pectoral** (Dial 1992). Este músculo tiene su origen en la superficie ventrolateral de la quilla y se inserta en la cresta deltoidea del húmero, del cual tira al contraerse (George & Berger 1966). Durante la elevación del ala es el ***supracoracoideus*** el músculo que juega un papel preponderante (Dial 1992). Pese a situarse también en una posición ventral, por debajo del pectoral, el *supracoracoideus* hace subir el ala mediante un sistema de polea. La existencia de un pequeño orificio, el canal trióseo, permite el paso del tendón que inserta a este músculo en la superficie superior del húmero, al cual levanta cuando se contrae (George & Berger 1966). Además de estos dos músculos, otros situados en la parte dorsal y en el ala están implicados de distinta forma en diferentes fases y tipos de vuelo.

Desde un punto de vista metabólico el vuelo es un tipo de locomoción eficiente si se considera por unidad de distancia recorrida, pero es energéticamente muy caro al considerarlo por unidad de tiempo empleada (Tucker 1968). Por todo ello, el conjunto formado por el pectoral y el *supracoracoideus* (*"tutta la carnosità di muscoli e polpe del petto"*, como lo denominó Leonardo) puede llegar a constituir hasta el 40% del peso total del ave (Hartman 1961). Desde un punto de vista estrictamente fisiológico hay que añadir que las aves presentan mayores gastos cardíacos (Grubb 1983) y corazones más grandes, los cuales, a iguales valores de consumo de oxígeno, tienen frecuencias cardíacas basales menores que las de mamíferos de pesos similares (Lasiewski & Calder 1971). Dado que los hábitos locomotores de un animal son un factor muy importante en su ecología y energética, cualquier adaptación al vuelo es el resultado de un compromiso entre una gran variedad de demandas impuestas sobre el diseño morfológico y fisiológico del animal (Rayner 1981). Como respuesta a estas demandas, las diferentes especies de aves han desarrollado estilos de vuelo específicos que implican distintos niveles de actividad. Desde un punto de vista teórico se pueden considerar seis tipos básicos (Viscor & Fuster 1987).

1. Vuelo de corta duración (*short flight*). Las especies con alas cortas y anchas y con un área alar pequeña generalmente sólo pueden desarrollar vuelos explosivos que implican cortos períodos de actividad. A este grupo pertenecen, por ejemplo, los Galliformes.

2. Vuelo cernido o estacionario (*hovering flight*). Mediante el continuo batido de las alas a una elevadísima frecuencia, algunas aves son capaces de mantenerse completamente estáticas en el aire. Este tipo de vuelo representa una especialización evolutiva que alcanza su máximo exponente en los colibrís.

3. Vuelo batido (*flapping flight*). Las especies que desarrollan este tipo de vuelo poseen elevadas frecuencias respiratorias y de batido de las alas. Son capaces de volar ininterrumpidamente durante largos períodos de tiempo y cubrir grandes distancias (migraciones). Son buenos ejemplos de este grupo las palomas, las fochas y la mayoría de las especies de anátidas.

4. Vuelo oscilatorio (*bounding flight*). Es de hecho una modalidad de vuelo batido que, debido a su elevado coste energético, alterna cortos períodos de vuelo ascendente y descendente. Durante el vuelo ascendente se baten vigorosamente las alas, mientras que en los períodos de vuelo descendente éstas permanecen pegadas al cuerpo. Todos los Paseriformes utilizan esta modalidad.

5. Vuelo ondulado (*undulating flight*). En este caso se alternan períodos de vuelo batido, durante los cuales se gana altura, con períodos de vuelo planeado que suponen un ahorro energético importante. Las urracas y algunas especies de pájaros carpinteros son grandes especialistas en este tipo de vuelo.

6. Vuelo planeado (*gliding* y *soaring flight*). Las gaviotas, albatros, buitres, águilas y otras muchas especies de aves generalmente de gran tamaño son capaces de permanecer largos períodos de tiempo volando sin necesidad de batir sus alas. Ello supone un gran ahorro energético y es posible gracias a sus grandes superficies alares.

4.2. Locomoción acuática

Un gran número de especies de aves dependen del medio acuático. El desarrollo de especializaciones en la musculatura de las patas y la presencia de membranas interdigitales en los pies constituyen algunos ejemplos de adaptaciones acuáticas específicas (Storer 1960). Dos comportamientos locomotores diferentes pueden registrarse en aves acuáticas.

1. La **natación** en aves como los ánades reales (*Anas platyrhynchos*) o los porrones moñudos (*Aythya fuligula*), es un modo de locomoción que requiere un incremento en el consumo de oxígeno de 2 a 4 veces el consumo basal (Prange & Schmidt-Nielsen 1970; Butler *et al.* 1988). El consumo de oxígeno durante la natación no es lineal y se mantiene relativamente constante dentro del rango de velocidades a las que generalmente nadan ánades y porrones, experimentando un incremento muy pronunciado más allá de este intervalo (Phillips *et al.* 1985). Estos valores de consumo de oxígeno son bajos si se comparan con los valores correspondientes al vuelo que, en el caso del porrón moñudo, superan en más de dos veces a los valores de la natación (Turner & Butler 1988), y en otras especies de aves oscilan entre 7 y 15 veces los consumos basales, según el peso del animal (Baudinette & Schmidt-Nielsen 1974; Bucher & Morgan 1989). Ahora bien, si se tiene en cuenta la masa de los músculos implicados en una y otra forma de locomoción, el consumo aeróbico es de un orden similar (Prange & Schmidt-Nielsen 1970), lo cual indica que la natación también es un medio de locomoción con elevadas demandas energéticas.

2. El **buceo** es empleado por muchas especies de aves que obtienen con esta forma de locomoción una parte o toda su fuente de alimento. El metabolismo energético del buceo es substancialmente elevado, como demuestran los datos obtenidos por Woakes & Butler (1983) en porrones moñudos. Estos autores estimaron un consumo de oxígeno que, bajo duraciones medias de buceo de 14 s, incrementaba en 3.5 veces el consumo del estado basal. Los estudios realizados en pingüinos, especies muy bien adaptadas al buceo, demuestran incrementos entre 4 y 7 veces la tasa metabólica basal con tiempos medios de inmersión de 2.3 minutos (véase Butler 1991). En general, el comportamiento más extendido es la realización de buceos aeróbicos de corta duración con el consumo de las reservas corporales de oxígeno que se reemplazan una vez que el ave sube a la superficie. Este comportamiento parece más eficiente que los buceos anaeróbicos más largos, los cuales requerirían mayores tiempos de recuperación en la superficie para restituir la deuda de oxígeno, y convertirían al exhausto animal en presa fácil de los depredadores (Phillips *et al.* 1985). Aún así, algunas especies de aves como el pingüino emperador (*Aptenodytes forsteri*) y el pingüino rey (*Aptenodytes patagonicus*) pueden bucear durante períodos de 2.5 a 9 minutos a profundidades de hasta 290 m (Kooyman *et al.* 1971, 1982). Aunque las adaptaciones fisiológicas al buceo en aves son muy variadas (véase Butler 1988, 1991), es interesante destacar que, en los pingüinos rey, recientemente se ha encontrado que durante largos buceos la temperatura corporal disminuye produciéndose así un descenso de tasa metabólica y un menor consumo de oxígeno, con lo cual se evita la anaerobiosis (Bevan *et al.* 1995).

4.3. Locomoción terrestre

Las aves presentan diferentes capacidades para desplazarse en tierra. Algunas especies, como los avestruces y los emús, son exclusivamente terrestres ya que han perdido la capacidad de volar y su único medio de locomoción, incluso para escapar de los depredadores, es la carrera. Otras, como los zampullines y somormujos, tienen serios problemas para desplazarse en tierra debido a la posición de sus patas que, como adaptación a la natación y al buceo, ocupan una posición muy retrasada en la estructura general del animal (Del Hoyo *et al.* 1992). Más allá de especializaciones como las que se acaban de ejemplificar, la mayor parte de las aves utiliza las patas en tierra, en mayor o menor medida, para desarrollar diferentes actividades. Incluso aves vinculadas al medio acuático y con excelentes habilidades para el vuelo, como las anátidas, las gaviotas y las fochas, son buenas andadoras y pasan ciertos períodos de tiempo en tierra, donde realizan una gran variedad de actividades (Cramp & Simmons 1977, 1980, 1985).

En estudios realizados con diferentes especies de aves no corredoras (como porrones, patos o pingüinos) se ha observado que el consumo máximo de oxígeno durante la locomoción terrestre es similar al consumo experimentado por mamíferos del mismo peso durante el mismo tipo de ejercicio (Phillips *et al.* 1985). Sin embargo, en las formas exclusivamente corredoras como el emú, se ha visto que estas aves son capaces de elevar su consumo de oxígeno hasta valores del orden de 11 veces su nivel basal (Grubb *et al.* 1983). A nivel muscular, este hecho es posible gracias a la presencia de un músculo gastrocnemio muy desarrollado, con una composición fibrilar carente de fibras lentas y capaz de satisfacer altas necesidades catabólicas (Patak & Baldwin 1993). En todos los casos en la locomoción terrestre, a diferencia de lo ocurrido durante el vuelo o en la locomoción acuática, se ha observado siempre una relación lineal entre el consumo de oxígeno y la velocidad de la carrera (Fedak *et al.* 1974; Phillips *et al.* 1985), que implica unos mecanismos de control específicos en los sistemas respiratorio y circulatorio durante este tipo de locomoción (véase Phillips *et al.* 1985).

Material y Métodos

1. Animales de experimentación

Para poner a punto la técnica histoquímica combinada mATPasa y AChE descrita en el artículo **II** se utilizó una especie de mamífero (cuatro ratas Sprague-Dawley). El resto de animales empleados fueron especies de aves. Las palomas (*Columba livia*), utilizadas en los estudios descritos en los artículos **I-III**, fueron capturadas en la ciudad de Barcelona y suministradas por personal del *Parc Zoològic de Barcelona*. Los siete ánades reales (*Anas platyrhynchos*) y las seis fochas comunes (*Fulica atra*), utilizados en los artículos **III**, **IV**, **V** y **VII**, se obtuvieron en el *Parc Natural del Delta de l'Ebre (Tarragona)* durante las temporadas de caza de los años 1992 y 1993. El personal técnico del citado parque natural supervisó la entrega de los animales por los cazadores, la cual se realizó siempre bajo el permiso nº 5605/92 de la *Direcció General d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya*. Las seis gaviotas patiamarillas (*Larus cachinnans*), utilizadas en los artículos **VI** y **VII**, fueron suministradas por técnicos del citado departamento en Abril de 1993, durante la campaña para el control de la población realizada en las *Illes Medes (Girona)*.

2. Obtención y procesado de muestras

Los músculos se diseccionaron enteros excepto el pectoral de las aves, del cual se obtuvo una muestra de su parte central. Las muestras obtenidas para los artículos **IV-VIII** fueron marcadas con la finalidad de poder identificar la orientación anatómica del fragmento de músculo, y de los cortes subsiguientes, durante el seccionado en el criostato. Para los músculos de la rata se adoptó la nomenclatura de Greene (1959), mientras que para los músculos de las aves se utilizó la de Vanden Berge (1979); esta última recomendada por el *International Committee on Avian Anatomical Nomenclature* (integrado como sección de la *World Association of Veterinary Anatomists*). El nombre de los músculos, junto con la abreviatura y una referencia al artículo donde se estudian en cada especie, se muestran en la Tabla 1 de esta sección.

Las muestras fueron congeladas en isopentano preenfriado a -160°C y almacenadas en nitrógeno líquido según las recomendaciones de Dubowitz & Brooke (1973). Los cortes histológicos, de un grosor de 14-25 µm según las características de los ensayos, se realizaron en un criostato Frigocut Reichert-Jung (Heidelberg, Alemania) entre -22°C y -20°C y se montaron en portaobjetos gelatinizados al 2% para facilitar la adherencia.

Tabla 1. Músculos estudiados en cada especie, abreviaturas y artículos donde se ha desarrollado la investigación.

Especie	Músculo	Abreviatura	Artículo
Rata			
	Soleus	SOL	II
	Extensor digitorum longus	EDL	II
Paloma			
	Pectoralis	PEC	I-III
	Triceps scapularis	TSC	I
	Extensor metacarpi radialis	EMR	I
	Pronator superficialis	PRN	I
	Iliotibialis cranialis	ITC	II, III
	Gastrocnemius lateralis	GLE	I
	Scapulohumeralis caudalis	SCH	III
	Brachialis	BRC	III
	Anterior latissimus dorsi	ALD	II,III
	Serratus metapatagialis	SMP	III
Ánade real			
	Anterior latissimus dorsi	ALD	II
Ánade real, focha y gaviota			
	Pectoralis	PEC	II, IV-VII
	Scapulohumeralis caudalis	SCH	IV-VII
	Triceps scapularis	TSC	IV-VII
	Extensor metacarpi radialis	EMR	IV-VII
	Iliotibialis cranialis	ITC	II, IV-VII
	Gastrocnemius lateralis	GLE	IV-VII

3. Técnicas histoquímicas utilizadas

En los últimos 30 años se han puesto a punto gran número de técnicas histoquímicas con la finalidad de tipificar las fibras musculares. Esta amplia variedad de ensayos puede ser dividida en tres grandes grupos (Brooke & Kaiser 1974).

a) El grupo denominado de los "enzimas oxidativos", entre los que se encontrarían las deshidrogenasas.

b) Los enzimas implicados en el mecanismo de contracción muscular: las ATPasas.

c) Los enzimas que se integran en las vías metabólicas glicolíticas, como la fosforilasa.

También las colinesterasas ocupan un lugar destacado como enzimas investigados en histoquímica, aunque para la tipificación de fibras han sido menos utilizadas. Los diferentes tipos de fibras musculares, junto con sus características de capilarización y morfometría, permiten conocer las propiedades metabólicas funcionales de cada músculo y relacionarlas con sus necesidades fisiológicas.

Antes de procesar los cortes histológicos según las diferentes técnicas, todos ellos fueron colocados en el mismo tampón fijador (Guth & Samaha 1970) el cual evita que los cortes se separen de los portaobjetos, a la vez que previene la aparición de arrugas en el tejido y la deformación del mismo.

A continuación se detallan los ensayos histoquímicos desarrollados en esta tesis. La falta de ensayos de enzimas del grupo (c) se ve compensada con el uso del método para una deshidrogenasa indicadora de la actividad glicolítica (α-GPDH). Se especifican en cada caso las bases histoquímicas del ensayo.

3.1. Succinato deshidrogenasa (SDH)

Método de Nachlas *et al.* (1957). En este ensayo los cortes histológicos son incubados en un medio tamponado que contiene, como substrato de la reacción, succinato de sodio. Una sal de tetrazolium, el Nitro-BT, se utiliza como indicador de la actividad deshidrogenasa del enzima. El Nitro-BT es soluble pero, al ser reducido como resultado de la reacción deshidrogenasa, se transforma en un compuesto insoluble de un color azul intenso denominado formazán. La naturaleza insoluble del formazán hace que éste precipite en el lugar dónde la reacción ha tenido lugar. La típica apariencia particulada (de coloración azul) que se observa en el interior de las fibras (**III**: Fig. 1*F*) demuestra la distribución del enzima SDH unido a la membrana interna mitocondrial. La presencia de grandes cantidades de este enzima indicará fibras de carácter oxidativo, mientras que la ausencia de deposición de formazán significará que la fibra en cuestión posee un metabolismo principalmente anaeróbico.

3.2. α-Glicerofosfato deshidrogenasa (α-GPDH)

Método de Wattenberg & Leong (1960). La incubación se realiza en un medio tamponado que contiene glicerofostato, el substrato de la reacción. El Nitro-BT es también utilizado en este caso, actuando de manera análoga a la descrita en el ensayo SDH. La presencia de actividad α-GPDH puede ser utilizada como un indicador indirecto de la capacidad glicolítica de la fibra muscular, ya que este enzima desempeña un papel clave en la lanzadera de glicerofostato (véase Reichmann & Pette 1984). Esta lanzadera es responsable de la transferencia de equivalentes de reducción desde el NADH citoplasmático a la cadena de transporte electrónico mitocondrial (Lehninger 1972).

3.3. Miosina adenosín-trifosfatasa (mATPasa)

Método de Brooke & Kaiser (1970). Esta reacción histoquímica se basa en el método original de Padykula & Herman (1955) para detectar la actividad de la Ca^{2+}-ATPasa miofibrilar. En el medio de incubación se coloca una solución tamponadora con $CaCl_2$ y el substrato de la reacción, el ATP en forma de sal disódica. La mATPasa, localizada en las miofibrillas de las fibras musculares, hidroliza la molécula de ATP liberando su grupo fosfato terminal, el cual se combina con el calcio presente en el medio de incubación para formar una sal insoluble. En las reacciones subsiguientes el calcio es reemplazado por

cobalto formando una sal que, a su vez, se reemplaza por sulfuro de amonio. El precipitado marrón oscuro formado, pone de manifiesto los lugares donde ha tenido lugar la hidrólisis del ATP. La preincubación a diferentes valores de pH, generalmente de 4 a 4,6 y de 10 a 11, inhibe selectivamente la actividad ATPasa. Ello permite clasificar las fibras musculares como de tipo I y de tipo II dependiendo de su carácter ácido estable/alcalino lábil o ácido lábil/alcalino estable, respectivamente (Brooke & Kaiser 1969). En aves, además, se ha podido identificar un tipo de fibra con carácter ácido estable/alcalino estable, al cual se ha denominado tipo III (Barnard *et al.* 1982). El artículo **III** de esta tesis trata con mayor detalle estas clasificaciones en la musculatura de ave.

3.4. Técnica ATPasa para la tinción de capilares

La presente investigación incluye una técnica ATPasa (artículo **I**) la cual, modificando el ensayo de Rosenblatt *et al.* (1987), permite visualizar de manera nítida y clara los capilares y las fibras musculares en una sección transversal de músculo esquelético. Las ventajas y mejoras que dicha técnica supone respecto al ensayo original serán comentadas en el apartado de **DISCUSIÓN**. En este apartado sólo se describen las bases de la reacción histoquímica. El método de Rosenblatt *et al.* (1987), que a su vez es una modificación del de Meijer (1970), difiere del método ATPasa de Brooke & Kaiser (1970) en el pH del medio de incubación (7,2 en vez de 9,4), donde además se colocan iones de plomo en forma de nitrato. Esta es justamente la diferencia más importante, ya que el plomo es el que determina la visualización de la actividad del enzima. Ello se produce a partir del sulfuro de plomo que precipita como consecuencia del tratamiento con sulfuro de amonio en la parte final del protocolo. Rosenblatt *et al.* (1987) diseñaron esta técnica como un método ATPasa más que permitía, en algunos músculos, poner de manifiesto la red capilar y el tipo de fibra simultáneamente. La modificación que se introduce en esta tesis evita la tinción de las fibras (para ello se ha utilizado la técnica de Brooke & Kaiser 1970) manifestando en cambio, de manera más nítida, la red capilar debido a la actividad ATPasa del endotelio. Al quedar, además, las fibras musculares claramente individualizadas, la técnica es muy útil para realizar contajes capilares y mediciones de diferentes parámetros fibrilares.

3.5. Técnica combinada mATPasa y AChE

También se incluye en esta tesis una técnica original combinada mATPasa y AChE (artículo **II**) que permite identificar, sobre un mismo corte histológico (transversal o longitudinal), el tipo de fibra y la frecuencia y estructura de sus terminales nerviosos. Este ensayo histoquímico combina la técnica de Brooke y Kaiser (1970) para la mATPasa y una modificación del método AChE de Toop (1976) para la demostración de la inervación motora. Como en el caso anterior, su validación y mejoras respecto a los ensayos originales serán comentadas también en el apartado de **DISCUSIÓN**, describiéndose a continuación las bases de la reacción histoquímica. Después de desarrollar el ensayo histoquímico de Brooke & Kaiser (1970), se incuban los cortes en una solución tamponada que contiene como substrato de la reacción una sal iodada de acetiltiocolina. En el medio de incubación también se añaden iones de cobre en forma de sal de sulfato. La acción del enzima libera la tiocolina que, al reaccionar con los iones de cobre, resulta en un complejo químico de cobre-tiocolina. En las posteriores etapas del protocolo este complejo reaccionará con

ferrocianuro potásico y nitrato de plata formándose ferrocianuro de cobre y sulfuro de plata, los cuales precipitan dando una coloración marrón en los lugares donde la actividad AChE ha tenido lugar, es decir, en los terminales nerviosos de la unión neuromuscular.

3.6. Sudan Black B

Método de Chiffelle & Putt (1951). Aunque esta no es una técnica basada en una reacción enzimática, su aplicación tiene interés desde el punto de vista metabólico, pues informa sobre la presencia o ausencia de lípidos, los cuales constituyen un substrato energético importante del catabolismo aeróbico en la fibra muscular. Los cortes se colocan en un medio con Sudan B disuelto en propilenglicol. El Sudan B es más soluble en los lípidos de las secciones del tejido muscular que en la solución de propilenglicol. Por este motivo, la reacción de tinción se produce como resultado del traspaso del colorante de la solución a los lípidos presentes en los cortes.

4. Obtención de resultados

4.1. Microfotografía

Una vez procesados, los cortes se montaron en una gota de glicerina y, en el plazo de una semana, se realizaron las microfotografías sobre las que se llevaron a cabo los contajes de capilares, fibras, terminales nerviosos y las mediciones de las distancias máximas de difusión y dimensiones fibrilares (áreas y perímetros). Todas las microfotografías fueron realizadas entre 80x y 200x aumentos, con un microscopio óptico Dialux Leitz (Wetzlar, Alemania) equipado con una cámara Wild MPS51 (Heerburg, Suiza). La calibración se realizó tomando una fotografía de un portaobjetos micrométrico a cada aumento al inicio y otra al final de cada carrete, con el fin de asegurar que no hubiera variaciones de ampliación durante el proceso de revelado.

Las densidades capilares (CD) y fibrilares (FD) fueron contadas en "ventanas" de tejido de $2 \cdot 10^5$ μm^2 (**I, IV-VI**). El número obtenido fue multiplicado por cinco para expresar el valor del parámetro por mm^2. Las mediciones fibrilares se obtuvieron con el software Sigma-Scan (v.3.9), Jandel Scientific (Erkrath, Alemania) sobre una tableta digitalizadora Calcomp 23180-4 (Anaheim, USA) conectada a un ordenador personal (**I** y **VII**). En el trabajo **VII**, al menos fueron medidas 40 fibras de cada tipo en cada campo, excepto cuando se detectó una presencia inferior a este número en algún tipo de fibra, caso en el que se contaron todas las fibras existentes. En la Figura 1 del artículo **VII** se muestra con detalle la distribución del muestreo de las 56.498 fibras medidas. Se calcularon además, para cada tipo de fibra, los índices CCA y CCP que indican, respectivamente, el número de capilares por cada 1.000 μm^2 de área y por cada 100 μm de perímetro. El cálculo se realizó dividiendo el número de capilares por fibra (NCF) entre el área transversal de la fibra (FCSA) o entre el perímetro (FPER) y multiplicando la cantidad resultante por 1.000 o 100, según el caso.

4.2. Nomenclatura fibrilar

El primer paso antes de describir y estudiar extensamente los músculos de las cuatro especies de aves utilizadas (**III-VII**) fue adoptar una nomenclatura que permitiera una tipificación de fibras adecuada. Como ya se ha indicado, la metodología de Brooke & Kaiser (1970) aplicada a la musculatura de aves (Barnard *et al.* 1982) clasifica las fibras musculares en tres tipos principales: tipo I, tipo II (que a su vez se subdivide en IIA y IIB) y tipo III. En el artículo **III**, esta ha sido la nomenclatura adoptada y en él se puede encontrar una descripción pormenorizada de los perfiles de cada tipo de fibra en varios músculos, locomotores y posturales, de la paloma. Sin embargo, en los artículos **IV-VII** se ha querido dar un enfoque ecofisiológico y comparado, para lo cual se creyó más adecuado utilizar la nomenclatura basada en Peter *et al.* (1972), ampliamente revisada en aves por Butler (1991), ya que refleja de una manera más "fisiológica" las propiedades de cada tipo de fibra. Por ello, y aunque hay un alto grado de correspondencia entre ambas clasificaciones, se podrá observar que en los citados artículos en ningún momento se habla de fibras de tipo I o II. En su lugar se consideran fibras lentas (*slow*) o rápidas (*fast*). Tampoco será posible encontrar subdivisiones de las fibras rápidas en IIA y IIB, sino que las fibras rápidas son consideradas oxidativas (FOG, *fast oxidative glycolytic*) o anaeróbicas (FG, *fast glycolytic*), a partir de los perfiles enzimáticos obtenidos mediante el resto de ensayos histoquímicos. En cuanto a las fibras lentas, siempre se han encontrado características oxidativas (SO, *slow oxidative*) asociadas a ellas, excepto en una zona muy concreta del pectoral de la focha donde se han denominado SW (*slow white*), por sus características anaeróbicas. Una aproximación esquemática y detallada de la nomenclatura seguida puede encontrarse en las tablas 1 de los artículos **IV-VI**.

4.3. Elección de las zonas de muestreo

Exceptuando algunos músculos, como el SOL de ratas adultas (Bigard *et al.* 1996) o el ALD de ciertas aves (Miyazaki *et al.* 1984; Hather & Hikida, 1988; Antonio & Gonyea 1993), la musculatura esquelética de todos los vertebrados posee en general una gran heterogeneidad (Peters 1989; Rome 1994). Este hecho plantea importantes problemas cuando se trata de describir las características de un músculo ya que, dependiendo del nivel y de la zona transversal del corte escogidos, se pueden obtener considerables diferencias en parámetros como la población fibrilar (López-Rivero *et al.* 1992*a*) o la densidad capilar (Romanul 1965).

Esta importante particularidad del tejido muscular ha sido necesariamente tenida en cuenta en este trabajo de investigación. En todos los casos la zona ecuatorial de cada músculo fue escogida para la obtención de los datos. En los artículos **I** y **II**, al tratarse de trabajos metodológicos sin finalidad descriptiva o comparativa, la elección de las zonas de muestreo o **"campos"** en cada músculo se consideró al azar. En el artículo **III**, la necesidad de contar y comparar frecuencias de inervación representativas para cada músculo, determinó que en los músculos pequeños como el ALD, BRC y el compartimento posterior del SMP los contajes fueran realizados en todo el músculo; mientras que en músculos mucho mayores, como el PEC, el ITC y el compartimento anterior del SMP, se tuviera que realizar un transecto a través del eje mayor de la sección transversal del

músculo. En los trabajos **IV-VII**, con el fin de describir de manera extensiva y rigurosa cada músculo y poder obtener resultados comparables entre las tres especies de aves estudiadas, se diseñó un protocolo de muestreo que dividió cada sección transversal en una cuadrícula de la cual se escogieron, dependiendo de cada músculo, diversos campos para su estudio. Dicho protocolo de muestreo está detalladamente explicado en la sección *Material and Methods* del artículo **IV** y ilustrado en las Figuras 1-3 (**IV**), Figuras 2-4 (**V** y **VI**) y Figura 1 (**VII**).

4.4. Estadística

Dado el enfoque comparado que esta investigación plantea, sobretodo en los cuatro últimos trabajos, fueron utilizados varios tests estadísticos con el objetivo de valorar las posibles diferencias entre especies en los parámetros musculares estudiados. La metodología estadística utilizada se resume de la siguiente manera:

a) En los casos en que fue pertinente comprobar la normalidad de la distribución de los datos se hizo mediante la utilización del test de Kolmogorov-Smirnov (tabla de Lilliefors).

b) Cuando fue necesario transformar los datos de porcentajes en distribuciones normales (**I** y **III**) se aplicó la función arcoseno según Sokal & Rohlf (1981):

$$\text{arcsen}\ \sqrt{\frac{x}{100}}$$

c) Para valorar si existían diferencias en la CD, FD y tamaño de las fibras entre los cortes histológicos procesados con la técnica de Rosenblatt *et al.* (1987) y los procesados con la modificación que se introduce en el artículo **I**, se utilizó el test t-Student de datos apareados, en el caso de distribuciones normales, y el test de rango-signo de Wilcoxon para datos apareados, en el caso de distribuciones no normales.

d) Los análisis de la varianza (ANOVA) de una vía se han utilizado para comprobar las diferencias estadísticas entre grupos de datos en los siguientes casos:

Para valorar si existían diferencias entre las frecuencias de inervación en un mismo tipo de fibra de músculos diferentes y, considerando todos los músculos juntos, si había diferencias en la frecuencia de inervación entre diferentes tipos de fibras (**III**).

Para valorar las diferencias morfométricas, a nivel global y en cada músculo en concreto, entre las tres especies salvajes, por un lado, y entre los tres tipos de fibras, por el otro (**VII**).

e) Los ensayos de ANOVA de dos vías, considerando como factores los campos y los individuos, se han utilizado en los artículos **IV-VI** con el fin de valorar las diferencias estadísticas en los parámetros %OFN, %OFA, CD, FD y C/F para cada músculo.

f) El método de Scheffé se utilizó como test *a posteriori* si, después de la aplicación de cada ANOVA, se observaron diferencias significativas. Este método es el más conservativo de todos los tests de comparaciones múltiples, es decir, es el más restrictivo cuando se trata de detectar diferencias significativas entre muestras (**III, IV-VII**).

g) Finalmente, para valorar las afinidades funcionales entre músculos diferentes de una misma especie (**IV-VI**) se aplicaron técnicas de clasificación jerárquica (*cluster analysis*), utilizando la distancia euclídea al cuadrado, el método del máximo y los parámetros CD, FD, %OFA y el porcentaje de área de fibras lentas como variables. En el artículo **VII**, se ha obtenido otro dendograma de características similares pero utilizando como variables los parámetros FCSA, FPER, MDD y NCF, con el fin de establecer las afinidades y diferencias funcionales entre los músculos de especies diferentes. Se escogió el método del máximo de entre los demás algoritmos de clasificación porque éste genera agrupaciones más cerradas, es decir, pocos grupos muy separados entre sí y con subgrupos afines muy compactos. Se consideró que esta característica podría ser adecuada para describir las diferencias funcionales, cuando éstas fueran evidentes, entre músculos y especies.

La presentación de los resultados obtenidos y su posterior discusión se realizará en dos partes. En una primera sección se comentarán las cuestiones metodológicas investigadas en los artículos **I** y **II**. En la segunda parte, más extensa, se describirán y discutirán los hallazgos obtenidos a partir de los estudios **III-VII**, aunque en algún momento se hará referencia a datos obtenidos en las publicaciones metodológicas.

Resultados

CUESTIONES METODOLÓGICAS

1. Demostración de la red capilar en la musculatura esquelética

Cuando los cortes histológicos se procesaron preincubándolos a 4°C se observó que, en determinados músculos, la elevada tinción histoquímica de algunas fibras enmascaraba los capilares circundantes. Concretamente, en los músculos EMR y PRN resultó imposible obtener un valor fiable de la CD o cualquier otra estimación capilar (**I**: Figs. 1 y 3). Ahora bien, si la preincubación se desarrollaba a una temperatura de 25°C, el contaje capilar se podía realizar sin ningún problema, debido a la extrema atenuación de la tinción mATPasa en las fibras musculares (**I**: Figs. 2 y 4). La preincubación a 25°C no permite tipificar fibras sobre el mismo corte donde se realiza el contaje capilar, pero proporciona unos cortes histoquímicos de alta calidad, donde obtener buenas estimaciones capilares y mediciones de parámetros fibrilares.

Se observó, además, que los músculos con poblaciones fibrilares donde la tinción ATPasa era débil presentaban capilares de dudosa localización y perímetros fibrilares mal definidos (**I**: Figs. 5, 7, 9 y 11), cuando eran procesados según la técnica original (T=4°C). Por ello se realizaron cortes seriados en 16 campos elegidos al azar de los músculos PEC, TSC y GLE de la paloma, preincubándolos a 4°C y 25°C. En los tres músculos, los contajes de CD y el parámetro C/F resultaron ser significativamente inferiores (aproximadamente un 10%) en los cortes donde se había aplicado la técnica original (**I**: Tabla 1). No obstante, estas diferencias no se pudieron poner de manifiesto ni en la FD ni en las dimensiones fibrilares, medidas como FCSA y FPER (**I**: Tablas 1 y 2). Con el fin de poder corregir los valores obtenidos a partir de la aplicación de la técnica original, T=4°C, se presenta una ecuación de regresión lineal que permite obtener los valores de la CD y el C/F para la técnica modificada, T=25°C (**I**: Figs. 13 y 14).

2. Demostración combinada del perfil mATPasa y la inervación de la fibra muscular

Se diseñó una técnica combinada que muestra, sobre el mismo corte histológico, el perfil mATPasa y las características del terminal nervioso de cada fibra muscular. Después de comprobarse que la técnica original de tinción mATPasa no se veía alterada por la subsiguiente aplicación del ensayo AChE, se modificaron los siguientes pasos en la reacción AChE:

a) Para adaptar la reacción a la musculatura de ave, la concentración de la acetiltiocolina en el medio de incubación tuvo que duplicarse y la duración de la incubación se prolongó a 30-45 minutos.

b) Para evitar la aparición de precipitados artefactuales, la concentración de $AgNO_3$ se redujo al 5%.

En los cortes longitudinales preincubados a pH ácido las inervaciones de las fibras de tipo II y III se pusieron de manifiesto de manera clara debido a la tinción mATPasa baja o moderada que poseen estas fibras (**II**: Figs. 1 y 5). Sin embargo, en las fibras de tipo I, la gran estabilidad de esta tinción a pH ácido enmascaraba en algunos casos la estructura del terminal nervioso. Por ello fue muy útil la preincubación a pH alcalino, donde la ausencia de tinción mATPasa en las fibras de tipo I permitió ver con claridad la actividad AChE (**II**: Fig. 2). Los cortes transversales también fueron de una gran utilidad para diferenciar entre un tipo y otro de inervación, ya que las grandes placas motoras de las fibras II se observaron rodeando casi totalmente a las fibras, mientras que los terminales de las fibras I o III aparecieron en forma de pequeños puntos mucho más discretos (**II**: Figs. 3 y 7).

FIBRAS Y PARÁMETROS MUSCULARES

1. Tipos de fibras

Las características histoquímicas de todos los tipos de fibras identificados en este estudio se encuentran descritas en las tablas 1 de los artículos **III-VI**. La distribución exacta de cada tipo de fibra en las tres especies salvajes se encuentra ampliamente descrita e ilustrada por campos en el ánade real (**IV**: Figs. 1-4), la focha (**V**: Figs. 2-5) y la gaviota (**VI**: Figs. 2-5). En el caso de la paloma sólo se determinó el rango de variación porcentual de cada tipo de fibra (**III**: Tabla 2).

1.1. Fibras lentas *(Slow)*

En los músculos de la paloma estudiados se pudieron identificar dos tipos diferentes de fibras lentas: fibras de tipo I y fibras de tipo III. En la tabla 1 y en las Figuras 1 y 2 del artículo **III** se describen e ilustran las características histoquímicas de ambos tipos. Las principales diferencias son:

a) *El perfil mATPasa.* En las fibras III la tinción mATPasa fue estable a pH ácido y alcalino mientras que en las fibras I esta estabilidad sólo se manifestó a pH ácido.

b) *El perfil SDH.* Esta tinción puede considerarse moderada en las fibras I del ITC y SCH (**III**: Fig. 1*I*), así como en las fibras III del ALD y BRC (**III**: Fig. 1*C*). No obstante, donde se detectó una mayor presencia de fibras I (en el compartimento posterior del SMP), la tinción SDH debe ser considerada como baja.

c) *La estructura del terminal nervioso.* En secciones longitudinales procesadas según la técnica combinada descrita en **II**, puede observarse que el terminal nervioso de las fibras I presentó una estructura punteada (*"little knobs"*, **III**: Fig. 2*C,G,H*), mientras que en el de las fibras III se constató la típica estructura arracimada de las fibras tónicas (*"en grappe"*, **III**: Fig. 2*A*).

d) *La distribución de la inervación.* El test de comparaciones múltiples por el método de Scheffé mostró diferencias significativas, con un nivel de $0,05 > P > 0,01$ (**III**: Tabla 5), entre las frecuencias de inervación de los tipos I y III. Las fibras III, además de tener frecuencias de inervación ligeramente mayores que las fibras I, presentaron valores del cociente media/varianza (**III**: Tabla 6) que parecen indicar que la distribución de los terminales nerviosos en las fibras I debe ser considerada aleatoria, mientras que en las fibras III es uniforme.

Las fibras de tipo III se encontraron como integrantes exclusivas de músculos que desarrollan un rol eminentemente postural: el ALD y el BRC. Las fibras de tipo I del SMP compartieron estas características pero, además, estas fibras se observaron en bajas proporciones (<15%) en músculos motores con poblaciones fibrilares mixtas, ITC y SCH (**III**: Tabla 2).

Conviene destacar que las fibras I encontradas en la musculatura de ave presentaron características muy distintas a las fibras de tipo I descritas en los músculos de mamíferos. El artículo **II** ilustra algunas de estas diferencias a nivel de los terminales nerviosos:

a) La estructura del terminal nervioso de las fibras I del SOL de rata, con la típica forma de herradura (*"en plaque"*), contrastó con la estructura en forma de puntos dispersos de las fibras I presentes en el ITC de la paloma (**II**: Figs. 1 y 2).

b) La inervación de las fibras I en las aves es múltiple, mientras que en la rata siempre se encontró un solo terminal por fibra muscular (**II**: Figs. 1 y 2).

c) La intensidad de la tinción AChE fue mucho menor en las fibras I de la paloma que en las de la rata. De hecho, para mostrar de manera clara los terminales nerviosos, los músculos de aves se tuvieron que incubar en el medio AChE durante un tiempo 3 o 4 veces superior al de los músculos de rata.

En ninguno de los seis músculos locomotores estudiados en las tres especies de aves salvajes (**IV-VII**) se encontraron fibras de tipo III (fibras lentas tónicas). No obstante, su presencia en músculos posturales como el ALD del ánade real (**II**: Fig. 8), mostró idénticas características histoquímicas al ALD de paloma. Se ha identificado, además, un tipo de fibra lenta, con perfil mATPasa de tipo I, en la parte más profunda del PEC de focha, cuyas características oxidativas fueron similares a las encontradas en el compartimento posterior del SMP de paloma. Estas fibras, presentes en proporciones del orden del 8% (**V**: Fig. 2), han sido denominadas fibras lentas blancas (SW, *slow white*) en referencia a sus características anaeróbicas (**V**: Fig. 5*C-E*).

Las demás fibras lentas, denominadas SO, fueron análogas a las fibras de tipo I descritas en los músculos de la paloma (**IV**: Fig. 4*A-D*; **V**: Fig. 5*A-B*; **VI**: Fig. 5*A-D*). Su distribución fue relativamente abundante en algunos campos (parte anterior del GLE), mientras que en otros se dieron en escasas proporciones (músculo EMR) o fueron inexistentes (músculos TSC y PEC). Es interesante resaltar que las fibras SO generalmente se encuentran situadas en zonas cercanas al hueso (músculos de la pata) o en las partes profundas del músculo (zonas ventrales del SCH), zonas que son predominantemente oxidativas.

1.2. Fibras rápidas *(Fast)*

En la musculatura esquelética de la paloma se diferenciaron dos subtipos de fibras rápidas o de tipo II según el ensayo mATPasa. Ambos tipos de fibras presentaron estabilidad frente a la preincubación alcalina e idénticas características en cuanto a la estructura del terminal nervioso, frecuencia de distribución y la presencia de una única placa motora por fibra (**III**: Tablas 1 y 5; Fig. 2*C-D*). Las diferencias se observaron en las tinciones SDH y mATPasa después de la preincubación ácida. En general las fibras IIA resultaron ser anaeróbicas mientras que a las IIB se les consideró principalmente un metabolismo oxidativo. A pesar de ello, en algunos músculos como el ITC y el SCH, una cierta proporción de fibras IIA presentaron tinciones SDH moderadas, a la vez que algunas fibras IIB desarrollaron tinciones SDH de una intensidad demasiado baja como para considerarlas fibras totalmente oxidativas (**III**: Tabla 1).

La distribución de las fibras de tipo II en cada músculo fue muy variable dependiendo de la región muscular estudiada. En la Tabla 2 del artículo **III** se muestra el nivel de variación porcentual de cada tipo de fibra. En el PEC la proporción de fibras IIA y IIB varió de la zona superficial a la zona profunda del músculo y en el resto de músculos la variación también se observó de las partes anteriores a las posteriores. Cabe destacar, además, que independientemente de la zona escogida del PEC y SMP, las fibras IIA (que en estos músculos son claramente anaeróbicas) se encontraron generalmente distribuidas en la periferia de los fascículos musculares. Esta particularidad no fue patente en los músculos ITC y SCH, cuyas fibras IIA se distribuyeron de una manera más uniforme dentro de los fascículos.

Las fibras rápidas identificadas en los artículos **IV-VII** se clasificaron en dos modalidades: fibras glicolíticas con metabolismo anaeróbico (FG) y fibras glicolíticas con metabolismo oxidativo (FOG). En algunos casos, como en el PEC del ánade real, se observaron correspondencias al 100% entre los subtipos IIA y IIB descritos en el artículo **III** y los subtipos FG y FOG de los artículos **IV-VII**. En otros músculos como el GLE, ITC o SCH esta correspondencia no siempre fue evidente. El caso más extremo se encontró en el PEC de la gaviota donde, si se considera la nomenclatura basada en el ensayo mATPasa, se pueden poner de manifiesto dos subtipos de fibras rápidas (IIA y IIB), aunque ambos deben ser considerados FOG por sus características oxidativas y glicolíticas. Por todo ello, y para evitar confusión en un tema tan controvertido como es el de la tipificación fibrilar, se optó por una clasificación "útil" desde un punto de vista fisiológico y ambiental. Así pues, se consideró, como ya ha sido apuntado en la sección de **MATERIAL Y MÉTODOS**, una nomenclatura de mamíferos (SO, FOG y FG) adaptada a la musculatura motora de aves.

Los dos tipos de fibras rápidas predominaron en los músculos locomotores estudiados (**IV**: Figs. 1-3; **V-VI**: Figs. 2-4). De hecho, su presencia fue exclusiva en algunos músculos y en la mayoría de campos estudiados. Las fibras FOG, de manera similar a las SO, se distribuyeron con frecuencia en las partes profundas y anatómicamente cercanas al hueso (ITC, GLE y SCH). Por el contrario, la fibras FG suelen encontrarse en zonas superficiales y abundaron más en localizaciones posteriores donde, como en el caso del TSC y el GLE, actúan con un momento de fuerza mayor (**IV**: Figs. 2 y 3; **V**: Figs. 2 y 4). También es destacable la peculiar distribución de ambos tipos de fibras en el PEC del ánade real (muy

parecida a la del PEC de la paloma) donde, en la parte ventral, se encuentra una gran cantidad de fibras FG que disminuye de manera espectacular hasta la parte profunda del músculo donde predominan las fibras FOG (**IV**: Fig. 1).

2. Morfometría de las fibras SO, FOG y FG de los músculos locomotores

Si se consideran las medias globales de los parámetros morfométricos (FCSA, FPER y MDD) en cada tipo de fibra se pueden observar algunos hechos remarcables. Los histogramas presentados en la Figura 2 del artículo **VII** muestran que, en todos los casos, en las fibras con metabolismo oxidativo (FOG y SO) siempre se registraron valores inferiores a los de las fibras anaeróbicas (FG). Cuando estos valores se contrastaron estadísticamente, como se muestra en las partes inferiores (*"Fibres"*) de las tablas que acompañan a cada histograma, se observaron diferencias significativas en los tres parámetros entre las fibras FOG y FG. La diferencia mayor se observó en los músculos del ánade real ($P \leq 0{,}001$), seguida de la focha ($0{,}01 \geq P > 0{,}001$) y de la gaviota ($0{,}05 \geq P > 0{,}01$). No se registraron diferencias significativas entre las fibras SO y FG de la gaviota, mientras en las otras dos especies se observó un nivel de significación bajo.

Cuando se consideró, a nivel global, el NCF se observaron valores muy similares entre los tres tipos de fibras, con una cierta tendencia, sólo significativa en el ánade real, a valores menores en las fibras FOG que en las fibras FG y SO (**VII**: Fig. 3*a*). No obstante, cuando se consideró el índice que describe el número de capilares en relación al área de la fibra, CCA (**VII**: Fig. 3*b*), se puso de manifiesto una clara diferencia estadística entre las fibras FOG y FG, reflejando un mayor número de capilares por área en las FOG. En el ánade real esta diferencia también fue altamente significativa para los valores del número de capilares por perímetro, CCP (**VII**: Fig. 3*c*).

3. Variabilidad individual e intramuscular

3.1. Individuos

Los resultados de los ANOVA de dos vías que se muestran en las tablas 4 de los estudios **IV-V** y en la tabla 5 del trabajo **VI** indican la existencia de una gran variabilidad entre individuos de la misma especie. Prácticamente en todos los músculos se detectaron diferencias estadísticas en alguno de los parámetros cuantificados (%OFN, %OFA, CD, FD y C/F).

3.2. Regiones musculares

También se pusieron de manifiesto diferencias entre campos del mismo músculo (**IV-V**: tablas 3; **VI**: tabla 4), resultando una gran variación regional, la cual es más patente en el ánade real y en la focha que en la gaviota. Esta regionalización muscular pudo ser

constatada a partir de los tests Scheffé de comparaciones múltiples mostrados en los artículos **IV-VI**. En el caso del ánade real y la focha, todos los músculos presentaron diferencias significativas entre campos en alguno de los parámetros oxidativos (especialmente en la CD y el %OFA). En la gaviota, por el contrario, el test Scheffé sólo puso de manifiesto una variación regional clara en el %OFA del músculo GLE. Los dendogramas presentados en los artículos **IV-VI** (Figs. 8 y 9) muestran las afinidades entre los diferentes campos estudiados de cada especie en relación a las variables CD, FD, %OFA y el porcentaje de área de fibras lentas.

Estas variaciones zonales descritas en el ánade real, la focha y la gaviota también pueden intuirse en el caso de la musculatura de la paloma. Aunque el objetivo del artículo **I** fue únicamente metodológico, como resultado del muestreo al azar se encontró, dentro de un mismo músculo, una gran variabilidad en los valores de CD, FD y C/F.

4. Resultados comparados de la composición fibrilar, capilarización y morfometría muscular

4.1. Morfometría general comparada

Los histogramas presentados en las Figuras 2 y 3 del artículo **VII** muestran una aproximación comparada de los valores morfométricos globales (considerando las medias de todos los campos de cada músculo). Como puede observarse en la parte superior (*Birds*) de las tablas anexas a dichas figuras, en los parámetros de medida directa (FCSA, FPER y MDD), sólo se detectaron diferencias significativas entre las fibras FOG de los ánades reales y las gaviotas. Sin embargo, al considerar el NCF por unidad de área o perímetro de fibra (CCA y CCP) las diferencias estadísticas fueron más evidentes entre fibras FOG y FG en las tres especies de aves, especialmente en el parámetro CCP (**VII**: Fig. 3*b,c*).

4.2. Composición de fibras en cada músculo

Se ha creído oportuno ilustrar gráficamente, en forma de histogramas, los valores del %OFN (presentados en las tablas 2 de los artículos **IV-VI**) con el fin de resumir la proporción de fibras oxidativas. La Figura 1 de esta sección permite comparar la capacidad oxidativa de los músculos entre las tres especies y muestra, a su vez, las variaciones regionales en cada caso. Es interesante resaltar que en los músculos TSC, SCH y GLE se obtuvo en las tres especies una arquitectura similar, aunque con claras diferencias cuantitativas.

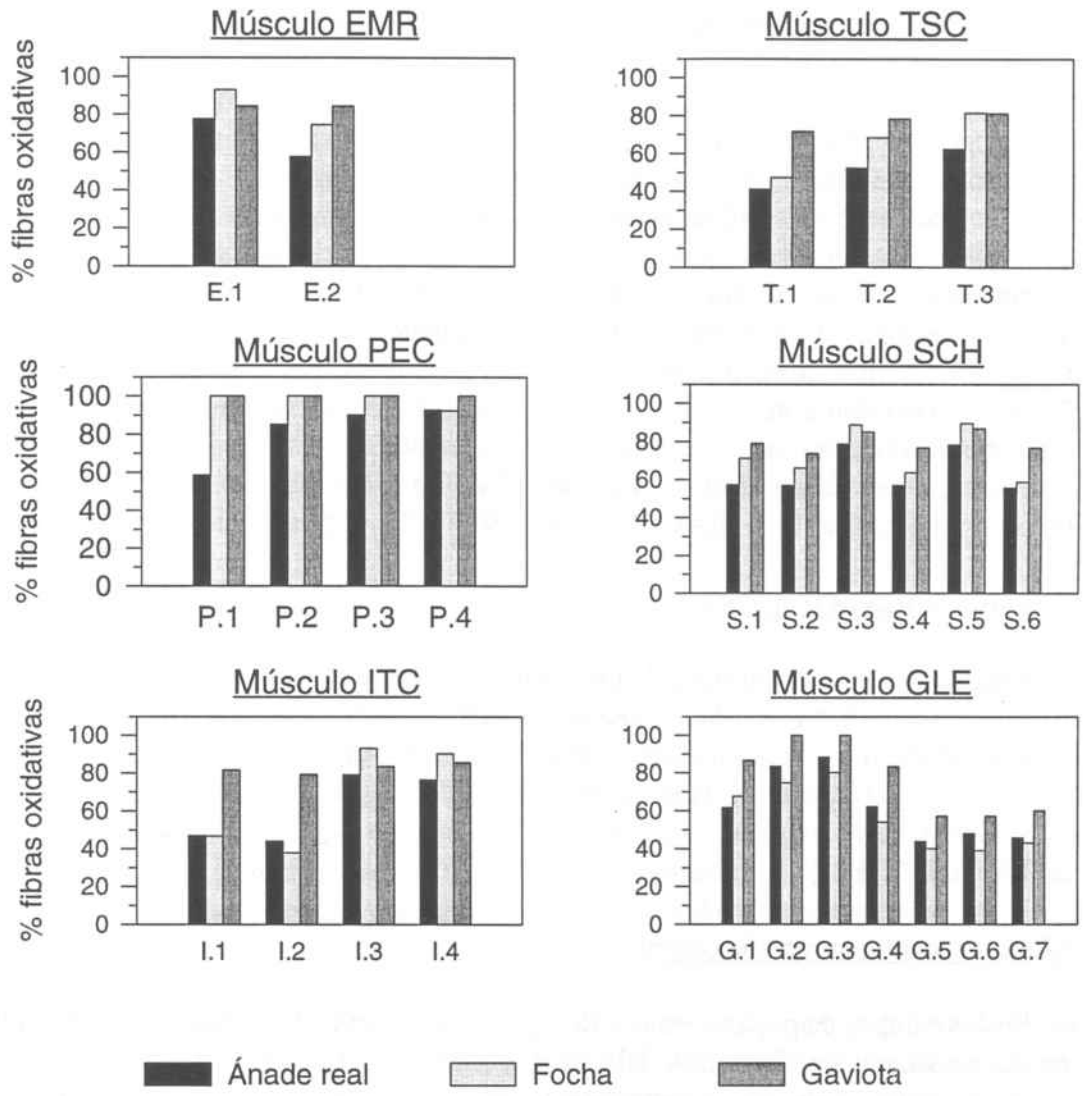

Figura 1. Porcentaje de fibras oxidativas en ánades reales, fochas y gaviotas. Los histogramas muestran los campos de cada músculo.

4.2.1. Músculos implicados en la locomoción aérea

Músculo extensor metacarpi radialis

En los EMR de focha y gaviota se pudo encontrar una baja proporción de fibras SO, aproximadamente del 4 al 9% (**V-VI**: Fig. 2), lo cual contrastó con la ausencia de fibras lentas en el ánade real (**IV**: Fig. 1). Este músculo se compone, en las tres especies, de dos compartimentos claramente separados. En la focha y el ánade real, la parte ventral presentó una mayor proporción de fibras FG que la parte dorsal (Fig. 1 de esta sección; **IV**: Fig. 1; **V**: Fig. 2), mientras que en la gaviota ambos compartimentos resultaron ser muy similares en proporciones fibrilares (Fig. 1; **VI**: Fig. 2).

Músculo pectoralis

Los resultados obtenidos en la proporción de fibras FOG indican que el PEC de gaviota y el de focha son músculos muy homogéneos en cuanto a capacidad oxidativa (Fig. 1; **V-VI**: Fig. 2). Estos datos contrastaron claramente con los obtenidos en el PEC del ánade real, donde se observó una gran presencia de fibras FG en las partes superficiales del músculo, la cual disminuyó considerablemente en los campos más profundos (Fig. 1; **IV**: Fig. 1). A pesar de las similitudes en la composición de fibras entre el PEC de la focha y el de la gaviota, hay también algunas claras diferencias. En primer lugar, la intensidad de la tinción SDH fue mucho mayor en las fibras FOG del PEC de focha que en el mismo tipo de fibras de la gaviota (**V**: Fig. 5*C*; **VI**: Fig. 5*G*), indicando una mayor actividad SDH en el PEC de focha. En segundo lugar, en la zona más profunda del PEC de focha apareció un 7,6% de fibras lentas anaeróbicas (**V**: Fig. 2, Fig. 5*C,D*) que no se observó en la gaviota. Finalmente, las fibras FOG del PEC de gaviota pudieron subdividirse en IIA y IIB de acuerdo con sus características mATPasa (**VI**: Fig. 5*H*, Tabla 1), con proporciones que oscilaron del 23% al 14% (IIA) y del 77% al 86% (IIB), según el campo muestreado.

Músculo triceps scapularis

Ninguna de las tres especies de aves estudiadas presentó fibras SO en el TSC. Se pudo constatar un gradiente de fibras FOG de la zona posterior a la anterior en las tres especies. Este gradiente fue mucho más marcado en el TSC del ánade real y de la focha (Fig. 1; **IV**: Fig. 2; **V**: Fig. 3) que en el de la gaviota (Fig. 1; **VI**: Fig. 3), aunque en esta última especie es suficiente para explicar la aparición de diferencias significativas entre campos en el %OFN y el %OFA (**VI**: Tabla 4).

Músculo scapulohumeralis caudalis

En los campos de la parte ventral (S.3 y S.5) del SCH de las tres especies se obtuvo una presencia similar de fibras SO, del orden del 10% al 15 %. Estas zonas ventrales fueron marcadamente oxidativas y contrastaron con las zonas dorsales o superficiales, con tendencias más anaeróbicas (Fig. 1). No obstante, los porcentajes de fibras FG y FOG presentaron algunas diferencias entre las tres especies en la mayoría de los campos. Se detectaron mayores cantidades de fibras FG en el SCH del ánade real (**IV**: Fig. 2), valores intermedios en la focha (**V**: Fig. 3) y porcentajes más bajos en la gaviota (**VI**: Fig. 3).

4.2.2. Músculos implicados en la locomoción terrestre y acuática

Músculo gastrocnemius lateralis (pars externa)

Se pudieron observar, en la parte anterior del GLE, diferencias en la proporción de fibras SO entre las tres especies. Concretamente, se obtuvo un mayor número de fibras SO en el GLE de la gaviota con una variación del 17% al 34% (**VI**: Fig. 4). En el ánade real y en la focha se constataron valores inferiores con variaciones del 4% al 25% y del 11% al 19%, respectivamente (**IV**: Fig. 3; **V**: Fig. 4). En la parte anterior del GLE de la gaviota no se encontraron fibras FG, mientras que en el ánade real y en la focha se distribuyeron en proporciones relativamente bajas (10%-30%). En contraste con esta parte anterior claramente oxidativa, la parte posterior presentó mayores proporciones de fibras FG (Fig.

1). De hecho, en el ánade real y en la focha el número de fibras FG de la parte posterior del GLE superó al número de fibras FOG (**IV**: Fig. 3; **V**: Fig. 4) mientras que en la gaviota, aunque predominaron las fibras FOG, se obtuvieron proporciones del orden del 40% (**VI**: Fig. 4).

Músculo iliotibialis cranialis

En este músculo se observó una diferencia considerable entre la gaviota y las otras dos especies. El ITC del ánade real y el de la focha presentaron dos partes claramente diferentes en cuanto a la proporción de fibras: una parte anterior, sin fibras SO y con porcentajes de fibras FG superiores al 50%; y otra posterior, con proporciones de fibras lentas del orden del 14% al 20% y bajas cantidades de fibras FG (**IV**: Fig. 3; **V**: Fig. 4). En el ITC de gaviota no se evidenció este tipo de regionalización ya que, a nivel del %OFN y de los demás parámetros oxidativos, este músculo se mostró muy homogéneo (Fig. 1). Además cabe destacar la presencia de fibras SO en ambas zonas del músculo (**VI**: Fig. 4).

4.3. Capilarización

Se ha creído conveniente, con fines comparativos, sintetizar en forma de tabla los valores de CD, FD y C/F de las tres especies, los cuales se describen por separado en los artículos **IV-VI**. La Tabla 2 resume dichos datos y permite constatar un gran número de paralelismos con los histogramas que se muestran en la Figura 1.

a) En primer lugar se podrá observar que, dentro de un mismo músculo, los campos que presentan CD más elevadas también muestran mayores proporciones de fibras oxidativas.

b) También es patente el hecho que, en general, el músculo que globalmente posee mayores CD y FD (fibras más pequeñas) es el PEC, aunque debe resaltarse que en algunas zonas concretas del GLE, ITC y EMR de ánade real y focha se pueden presentar valores iguales o incluso mayores que en el PEC.

c) A nivel general, cabe destacar que las CD más altas se obtuvieron en los músculos de la focha y las más bajas en los del ánade real. La gaviota presenta valores intermedios. Esto contrasta con los datos de la Figura 1, donde las mayores proporciones de fibras oxidativas se encuentran generalmente en los campos de los músculos de la gaviota. Esta situación es especialmente paradójica en algunos casos concretos que se describen a continuación:

En la mayor parte de los campos de los músculos implicados en el vuelo: EMR, TSC, PEC y SCH. Mientras que las proporciones de fibras oxidativas son mayores en la gaviota (Fig. 1), las CD son bastante superiores en la focha (Tabla 2).

En los campos anteriores del GLE. El porcentaje de fibras oxidativas es también superior en la gaviota (Fig. 1), pero con valores de CD inferiores a los del ánade real (Tabla 2).

Tabla 2. Densidad capilar (CD), densidad de fibras (FD) y cociente entre el número de capilares y fibras (C/F) en cada campo para las tres especies de aves salvajes estudiadas: ánade real, focha común y gaviota patiamarilla. Medias expresadas con los límites de confianza al 95%.

	Ánade real			Focha			Gaviota		
	CD	FD	C/F	CD	FD	C/F	CD	FD	C/F
E.1	1064 ± 101	696 ± 54	1.53 ± 0.11	1660 ± 180	732 ± 167	2.33 ± 0.41	1182 ± 58	497 ± 82	2.42 ± 0.33
E.2	878 ± 100	597 ± 42	1.47 ± 0.15	1326 ± 169	546 ± 78	2.44 ± 0.24	1156 ± 180	520 ± 124	2.24 ± 0.62
T.1	814 ± 91	449 ± 53	1.91 ± 0.18	1042 ± 183	510 ± 99	2.06 ± 0.19	1119 ± 271	554 ± 147	2.05 ± 0.47
T.2	913 ± 94	488 ± 57	1.88 ± 0.20	1215 ± 153	593 ± 91	2.07 ± 0.30	1039 ± 133	497 ± 116	2.13 ± 0.39
T.3	1063 ± 89	606 ± 87	1.78 ± 0.20	1371 ± 173	678 ± 123	2.05 ± 0.27	1046 ± 202	504 ± 130	2.11 ± 0.42
P.1	768 ± 140	475 ± 147	1.68 ± 0.24	1579 ± 169	832 ± 76	1.90 ± 0.11	1233 ± 113	650 ± 94	1.91 ± 0.17
P.2	925 ± 41	717 ± 105	1.31 ± 0.17	1571 ± 184	861 ± 134	1.84 ± 0.20	1210 ± 143	665 ± 76	1.83 ± 0.21
P.3	1113 ± 93	793 ± 198	1.47 ± 0.31	1539 ± 236	829 ± 90	1.86 ± 0.23	1087 ± 98	614 ± 106	1.80 ± 0.28
P.4	1339 ± 121	930 ± 230	1.51 ± 0.31	1541 ± 320	930 ± 168	1.79 ± 0.14	1126 ± 131	589 ± 105	1.95 ± 0.32
S.1	768 ± 54	461 ± 47	1.68 ± 0.14	1028 ± 186	491 ± 100	2.10 ± 0.12	931 ± 122	491 ± 101	1.93 ± 0.28
S.2	716 ± 77	442 ± 86	1.65 ± 0.24	925 ± 113	453 ± 37	2.04 ± 0.16	929 ± 57	489 ± 92	1.94 ± 0.28
S.3	1065 ± 140	537 ± 57	1.99 ± 0.20	1229 ± 213	592 ± 88	2.09 ± 0.27	1056 ± 117	474 ± 100	2.29 ± 0.45
S.4	739 ± 120	442 ± 80	1.68 ± 0.09	1002 ± 174	532 ± 83	1.88 ± 0.13	924 ± 95	519 ± 76	1.80 ± 0.22
S.5	1091 ± 114	589 ± 85	1.89 ± 0.25	1259 ± 202	583 ± 80	2.16 ± 0.13	994 ± 98	505 ± 72	2.00 ± 0.31
S.6	773 ± 115	492 ± 69	1.57 ± 0.11	1001 ± 225	546 ± 107	1.83 ± 0.10	980 ± 105	537 ± 55	1.83 ± 0.14
I.1	908 ± 130	533 ± 64	1.70 ± 0.10	1109 ± 137	550 ± 61	2.02 ± 0.10	1062 ± 104	644 ± 59	1.65 ± 0.18
I.2	807 ± 84	503 ± 76	1.63 ± 0.19	1046 ± 191	555 ± 53	1.89 ± 0.31	1032 ± 100	636 ± 78	1.63 ± 0.23
I.3	1231 ± 138	638 ± 73	1.94 ± 0.20	1450 ± 140	716 ± 105	2.05 ± 0.32	1094 ± 183	626 ± 93	1.75 ± 0.11
I.4	1169 ± 134	620 ± 71	1.89 ± 0.12	1455 ± 262	686 ± 111	2.13 ± 0.32	1137 ± 202	643 ± 75	1.77 ± 0.21
G.1	871 ± 131	413 ± 57	2.13 ± 0.35	1090 ± 140	498 ± 92	2.23 ± 0.39	940 ± 78	456 ± 62	2.08 ± 0.22
G.2	1230 ± 151	492 ± 92	2.55 ± 0.37	1159 ± 111	503 ± 117	2.36 ± 0.35	1066 ± 200	534 ± 164	2.06 ± 0.33
G.3	1385 ± 267	490 ± 80	2.89 ± 0.67	1091 ± 125	479 ± 83	2.31 ± 0.32	1017 ± 294	510 ± 175	2.04 ± 0.29
G.4	947 ± 84	427 ± 54	2.24 ± 0.24	936 ± 57	436 ± 63	2.18 ± 0.32	945 ± 114	522 ± 119	1.85 ± 0.29
G.5	728 ± 96	386 ± 53	1.91 ± 0.30	824 ± 81	405 ± 85	2.07 ± 0.27	879 ± 188	500 ± 122	1.78 ± 0.20
G.6	760 ± 40	389 ± 44	1.98 ± 0.24	817 ± 72	417 ± 45	1.97 ± 0.19	816 ± 88	532 ± 113	1.56 ± 0.19
G.7	756 ± 72	390 ± 62	1.97 ± 0.28	807 ± 87	423 ± 71	1.93 ± 0.25	865 ± 103	510 ± 146	1.76 ± 0.33

d) En ambos casos, los valores inferiores de CD obtenidos en los músculos de la gaviota pueden ser debidos, al menos en parte, a las bajas FD que estos músculos presentan en relación a los de la focha y el ánade real (Tabla 2).

e) Finalmente conviene resaltar que, a pesar de las diferencias mencionadas, los valores de C/F encontrados fueron bastante similares en los campos a los que se ha hecho referencia (Tabla 2).

Los gráficos cartesianos que muestran las relaciones entre la FD y CD (**IV**: Fig. 5; **V-VI**: Fig. 6) ponen de manifiesto algunas características interesantes sobre la distribución de estos parámetros en los seis músculos estudiados. En la gaviota, se observa una concentración de puntos en una pequeña zona de la gráfica con rectas de regresión que carecen de un patrón común (**VI**: Fig. 6). Esto contrasta con la gran dispersión de puntos observada en las gráficas del ánade real y la focha (**IV**: Fig. 5; **V**: Fig. 6). No obstante, entre la focha y el ánade real se encontró una diferencia notable. En todos los músculos de la focha, la FD y CD variaban de manera parecida resultando rectas de regresión que ofrecen, en cada músculo, pendientes similares que tienden a solaparse (**V**: Fig. 6). Por el contrario, en el ánade real estos cambios fueron muy diferentes dependiendo del músculo estudiado, dando lugar a rectas de regresión con pendientes muy desiguales (**IV**: Fig. 5).

4.4. Morfometría de cada músculo

Para cada parámetro medido en el estudio **VII** se presenta una tabla donde se resumen los resultados obtenidos en las tres especies. De esta manera se sintetizan a continuación, con fines comparativos, los valores de FCSA (Tabla 3), FPER (Tabla 4), MDD (Tabla 5) y NCF (Tabla 6) en cada tipo de fibra y para cada especie. Las Figuras 5 y 6 del artículo **VII** amplían la información contenida en las tablas aquí presentadas desde un punto de vista estadístico, incorporando los ANOVA y los tests de comparaciones múltiples para cada tipo de fibra y músculo. Los tres dendogramas (uno para cada tipo de fibra) presentados en la Figura 4 del trabajo **VII** muestran las afinidades entre los diferentes músculos de cada especie con respecto a las variables FCSA, FPER, MDD y NCF.

4.4.1 Fibras SO

En los GLE de las tres especies las características morfométricas de las fibras SO no presentaron ninguna diferencia estadística (**VII**: Fig. 5*a*). Sin embargo, en el ITC, el otro músculo de la pata estudiado, se obtuvieron valores de FCSA, FPER y MDD significativamente más bajos en el ánade real que en las otras dos especies (Tablas 3-5 de esta sección; **VII**: Fig. 5*d*). En cuanto a los músculos implicados en el vuelo con presencia de fibras lentas (EMR de focha y gaviota y SCH de las tres especies), se observaron en ambos casos fibras SO mucho mayores en la gaviota que en el ánade real y la focha (Tablas 3-5). Cuando fue posible aplicar un test de comparaciones múltiples, en el caso del SCH, estas diferencias resultaron ser significativas (**VII**: Fig. 6*a*).

4.4.2. Fibras FG

Se encontró una tendencia marcadamente opuesta entre las fibras anaeróbicas de los músculos implicados en el vuelo y los de la pata. En concreto, no se detectaron diferencias estadísticas de tamaño entre las fibras FG de ninguno de los músculos del ala (**VII**: Fig. 6*b,d,f*), mientras que se obtuvieron valores con grandes diferencias significativas entre las tres especies en el GLE (**VII**: Fig. 5*b*) y entre el ánade real y la gaviota en el ITC (**VII**: Fig. 5*e*). En todos estos casos, las fibras FG de los músculos de la pata fueron considerablemente mayores en el ánade real y en la focha que en la gaviota (Tablas 3-5). Por esta razón, las fibras FG de los músculos GLE del ánade real y la focha quedan totalmente segregadas a una gran distancia de las de la gaviota en el dendograma del artículo **VII** (Fig. 4*c*).

4.4.3. Fibras FOG

En las fibras aeróbicas se observó una tendencia inversa a la descrita para las fibras FG. En el GLE no se observaron diferencias significativas de tamaño entre fibras FOG de especies diferentes (**VII**: Fig. 5*c*) y éstas fueron relativamente pequeñas en el ITC (**VII**: Fig. 5*f*). Esta similitud entre especies en la morfometría de las fibras FOG de los músculos de la pata contrasta con las diferencias, estadísticamente muy significativas, que se obtuvieron en los músculos del ala. Destaca por su alta significación el PEC (**VII**: Fig. 6*h*) donde las fibras FOG del ánade real y la focha presentaron tamaños considerablemente menores que las de la gaviota (Tablas 3-5). En el SCH y el TSC, aunque de manera menos espectacular, también se pudo observar la presencia de fibras FOG mayores en la gaviota que en las otras dos especies (**VII**: Fig. 6*c,e*).

Como características comunes a las tres especies cabe señalar las importantes diferencias de tamaño entre las fibras FOG del GLE y del ITC (Tablas 3-5). Esto se ve reflejado en el dendograma de la Figura 4*b*, donde los GLE de las tres especies quedan segregados a una gran distancia de los ITC.

Tabla 3. Área de la sección transversal de la fibra (FCSA) en cada campo para las tres especies de aves salvajes estudiadas: ánade real, focha común y gaviota patiamarilla. Medias expresadas en μm^2 con los límites de confianza al 95%..

	Ánade real			Focha			Gaviota		
	SO	FOG	FG	SO/SW*	FOG	FG	SO	FOG	FG
E.1		1002 ± 125	2093 ± 259	1327 ± 250	1224 ± 372	1698 ± 540	2553 ± 508	1789 ± 350	2309 ± 516
E.2		1014 ± 98	1873 ± 164	1376 ± 239	1646 ± 320	2008 ± 390	1944 ± 233	1840 ± 320	2376 ± 438
T.1		1061 ± 87	2578 ± 265		1378 ± 327	2398 ± 515		1570 ± 365	2155 ± 608
T.2		1052 ± 117	2525 ± 310		1349 ± 341	2133 ± 563		1738 ± 383	2304 ± 355
T.3		1082 ± 180	2451 ± 289		1242 ± 292	1746 ± 353		1835 ± 410	2442 ± 563
P.1		850 ± 169	2742 ± 378		1073 ± 102			1501 ± 311	
P.2		878 ± 115	2584 ± 285		1069 ± 190			1437 ± 164	
P.3		773 ± 159	2238 ± 512		1030 ± 156			1500 ± 277	
P.4		756 ± 170	1915 ± 327	1608 ± 419*	984 ± 192			1550 ± 233	
S.1		1135 ± 154	2788 ± 500		1632 ± 205	2612 ± 265		1606 ± 258	2357 ± 492
S.2		1273 ± 177	3109 ± 460		1688 ± 129	2354 ± 156		1601 ± 282	2485 ± 422
S.3	1884 ± 507	1268 ± 220	2678 ± 434	1868 ± 311	1462 ± 151	2149 ± 274	2523 ± 496	1663 ± 340	2396 ± 411
S.4		1210 ± 217	2897 ± 327		1499 ± 150	2397 ± 282		1580 ± 262	2357 ± 389
S.5	1743 ± 363	1188 ± 194	2207 ± 309	1770 ± 227	1470 ± 123	2080 ± 447	2336 ± 498	1537 ± 364	2340 ± 385
S.6		1006 ± 133	2458 ± 142		1339 ± 105	2202 ± 165		1423 ± 184	2206 ± 251
I.1	1160 ± 118	1094 ± 112	2160 ± 205		1316 ± 301	1952 ± 398	1718 ± 271	1284 ± 185	1538 ± 163
I.2		1218 ± 145	2441 ± 391		1240 ± 237	1844 ± 243	1689 ± 219	1352 ± 202	1609 ± 241
I.3	1432 ± 234	1195 ± 192	1925 ± 280	1657 ± 321	1127 ± 248	1795 ± 466	1852 ± 310	1334 ± 283	1526 ± 186
I.4	1405 ± 208	1171 ± 205	1892 ± 245	1617 ± 375	1112 ± 275	1758 ± 386	1733 ± 256	1305 ± 275	1498 ± 216
G.1	2066 ± 157	1767 ± 307	3134 ± 487	1373 ± 480	1649 ± 455	2595 ± 687	1968 ± 346	1851 ± 128	2347 ± 189
G.2	1789 ± 552	1735 ± 312	2639 ± 532	1477 ± 358	1662 ± 438	2452 ± 724	1853 ± 410	1704 ± 373	
G.3	1911 ± 372	1746 ± 248	2531 ± 449	1738 ± 467	1769 ± 275	2510 ± 366	2037 ± 459	1748 ± 447	
G.4	1403 ± 103	1614 ± 281	3171 ± 595		1825 ± 371	2549 ± 405	1806 ± 377	1698 ± 303	2151 ± 453
G.5		1595 ± 338	3050 ± 603		1867 ± 343	2764 ± 514		1711 ± 289	2114 ± 393
G.6		1610 ± 309	3132 ± 518		1809 ± 127	2567 ± 187		1623 ± 247	1987 ± 344
G.7		1615 ± 301	3181 ± 578		1897 ± 353	2597 ± 396		1684 ± 327	2079 ± 399

Tabla 4. Perímetro de la fibra (FPER) en cada campo para las tres especies de aves salvajes estudiadas: ánade real, focha común y gaviota patiamarilla. Medias expresadas en μm con los límites de confianza al 95%.

	Ánade real			Focha			Gaviota		
	SO	FOG	FG	SO/SW*	FOG	FG	SO	FOG	FG
E.1		119 ± 7	173 ± 12	139 ± 14	132 ± 19	156 ± 23	192 ± 17	161 ± 16	184 ± 22
E.2		119 ± 6	165 ± 8	139 ± 12	153 ± 14	173 ± 13	170 ± 11	164 ± 13	187 ± 15
T.1		123 ± 6	191 ± 11		140 ± 17	186 ± 21		151 ± 19	178 ± 28
T.2		121 ± 7	191 ± 13		134 ± 13	170 ± 21		156 ± 19	179 ± 15
T.3		123 ± 10	181 ± 10		130 ± 8	157 ± 14		162 ± 19	188 ± 25
P.1		111 ± 11	201 ± 14		122 ± 6			147 ± 16	
P.2		111 ± 8	195 ± 11		122 ± 11			143 ± 7	
P.3		103 ± 11	180 ± 20		119 ± 9			146 ± 12	
P.4		102 ± 12	166 ± 15	148 ± 19*	117 ± 11			148 ± 10	
S.1		127 ± 10	200 ± 18		154 ± 10	197 ± 9		152 ± 9	184 ± 16
S.2		134 ± 11	213 ± 16		155 ± 6	201 ± 6		151 ± 13	189 ± 18
S.3	161 ± 21	135 ± 12	199 ± 15	165 ± 16	148 ± 8	179 ± 10	187 ± 18	155 ± 14	187 ± 16
S.4		131 ± 12	204 ± 13		148 ± 9	187 ± 11		152 ± 12	188 ± 17
S.5	159 ± 18	131 ± 11	186 ± 15	160 ± 10	148 ± 5	175 ± 17	181 ± 18	150 ± 17	190 ± 15
S.6		119 ± 9	188 ± 5		139 ± 5	180 ± 7		144 ± 10	180 ± 13
I.1	130 ± 6	124 ± 7	176 ± 10		134 ± 17	170 ± 18	154 ± 14	136 ± 9	150 ± 8
I.2		131 ± 9	186 ± 17		132 ± 13	166 ± 12	154 ± 13	141 ± 11	152 ± 11
I.3	143 ± 11	130 ± 11	169 ± 14	155 ± 16	129 ± 15	163 ± 19	162 ± 15	140 ± 16	150 ± 11
I.4	142 ± 11	130 ± 12	168 ± 12	151 ± 18	127 ± 16	161 ± 22	156 ± 12	137 ± 15	147 ± 11
G.1	171 ± 6	159 ± 16	218 ± 22	143 ± 25	153 ± 20	193 ± 25	170 ± 15	166 ± 6	191 ± 11
G.2	160 ± 25	158 ± 16	200 ± 23	146 ± 18	155 ± 20	188 ± 26	163 ± 21	157 ± 19	
G.3	166 ± 17	159 ± 11	193 ± 19	158 ± 21	160 ± 12	193 ± 15	170 ± 21	160 ± 22	
G.4	143 ± 7	152 ± 13	213 ± 21		160 ± 16	194 ± 15	161 ± 19	158 ± 16	178 ± 19
G.5		150 ± 16	209 ± 23		161 ± 15	200 ± 19		157 ± 14	176 ± 17
G.6		150 ± 15	211 ± 18		160 ± 5	193 ± 7		153 ± 13	172 ± 15
G.7		150 ± 14	213 ± 18		163 ± 15	194 ± 15		157 ± 17	175 ± 18

Tabla 5. Distancias máximas de difusión (MDD) en cada campo para las tres especies de aves salvajes estudiadas: ánade real, focha común y gaviota patiamarilla. Medias expresadas en μm con los límites de confianza al 95%.

	Ánade real			Focha			Gaviota		
	SO	FOG	FG	SO/SW*	FOG	FG	SO	FOG	FG
E.1		19.5 ± 1.5	28.1 ± 1.9	22.0 ± 1.7	20.7 ± 1.8	24.8 ± 4.5	32.0 ± 2.0	26.1 ± 2.2	30.6 ± 3.3
E.2		19.4 ± 1.3	26.6 ± 1.0	21.4 ± 1.6	24.3 ± 1.5	26.2 ± 1.4	28.8 ± 1.5	27.3 ± 2.1	31.6 ± 3.3
T.1		19.7 ± 1.5	30.4 ± 1.4		22.7 ± 3.3	28.9 ± 2.7		24.6 ± 3.0	28.4 ± 4.0
T.2		20.3 ± 1.2	31.3 ± 2.1		21.3 ± 1.4	26.0 ± 2.4		25.0 ± 2.4	28.9 ± 1.7
T.3		20.5 ± 2.0	30.5 ± 1.6		20.7 ± 1.2	24.7 ± 1.5		25.9 ± 2.7	30.1 ± 4.0
P.1		18.4 ± 2.0	33.1 ± 1.8		19.6 ± 1.1			24.4 ± 2.2	
P.2		18.5 ± 1.7	32.4 ± 1.8		19.6 ± 1.9			24.0 ± 1.4	
P.3		16.8 ± 2.2	29.7 ± 3.1		19.5 ± 1.4			24.3 ± 2.2	
P.4		16.7 ± 2.1	26.7 ± 2.4	23.4 ± 3.2*	18.9 ± 1.9			24.2 ± 2.1	
S.1		20.8 ± 2.1	32.4 ± 2.6		24.6 ± 1.4	30.2 ± 1.2		25.5 ± 1.6	29.9 ± 2.4
S.2		22.5 ± 1.6	35.2 ± 2.9		24.9 ± 0.9	30.5 ± 1.6		25.3 ± 2.0	30.2 ± 2.8
S.3	26.7 ± 3.9	22.6 ± 2.5	32.4 ± 2.3	25.5 ± 2.8	23.1 ± 1.4	28.5 ± 1.3	30.3 ± 2.2	25.8 ± 1.7	30.7 ± 1.8
S.4		21.9 ± 1.8	33.6 ± 2.6		23.3 ± 1.1	29.0 ± 2.0		25.1 ± 2.0	30.6 ± 2.9
S.5	25.3 ± 2.0	22.0 ± 2.4	29.0 ± 2.0	25.4 ± 1.3	23.2 ± 1.2	27.8 ± 2.2	29.1 ± 2.2	25.2 ± 2.1	31.0 ± 2.6
S.6		19.2 ± 2.5	30.4 ± 1.5		22.5 ± 1.1	27.4 ± 1.2		24.4 ± 1.7	29.1 ± 1.9
I.1	20.7 ± 2.8	20.7 ± 1.4	29.0 ± 1.0		22.0 ± 2.4	26.7 ± 2.5	25.5 ± 2.1	22.1 ± 1.8	24.2 ± 1.7
I.2		21.5 ± 1.8	30.5 ± 2.3		21.0 ± 2.2	25.7 ± 1.0	24.3 ± 1.2	22.9 ± 1.9	24.5 ± 2.1
I.3	23.0 ± 2.7	21.9 ± 2.0	26.9 ± 2.0	24.1 ± 1.7	20.7 ± 2.6	25.5 ± 3.3	26.0 ± 2.2	22.7 ± 2.1	24.1 ± 2.3
I.4	22.9 ± 2.2	22.0 ± 3.4	26.4 ± 1.1	23.6 ± 2.0	20.1 ± 2.1	25.8 ± 3.8	25.4 ± 2.2	22.0 ± 2.5	24.3 ± 2.3
G.1	29.4 ± 1.8	26.2 ± 3.1	34.5 ± 1.6	22.3 ± 2.9	24.6 ± 2.3	29.5 ± 2.4	27.8 ± 2.3	27.1 ± 1.0	30.3 ± 1.3
G.2	25.7 ± 3.2	25.5 ± 2.1	32.6 ± 3.5	23.9 ± 2.8	25.2 ± 2.5	29.5 ± 3.0	26.6 ± 2.8	26.1 ± 2.6	
G.3	27.4 ± 2.5	27.1 ± 1.4	31.2 ± 3.2	25.1 ± 2.7	25.5 ± 1.5	30.5 ± 2.5	27.6 ± 2.5	26.0 ± 2.9	
G.4	23.4 ± 1.7	25.4 ± 2.0	34.4 ± 3.7		26.3 ± 2.3	30.2 ± 2.6	26.4 ± 2.0	25.9 ± 1.5	29.2 ± 2.7
G.5		24.8 ± 3.3	33.8 ± 3.4		26.6 ± 2.1	30.9 ± 2.7		25.9 ± 1.7	28.4 ± 2.2
G.6		25.0 ± 2.6	33.7 ± 2.1		25.9 ± 0.7	30.6 ± 1.9		25.5 ± 0.8	28.1 ± 1.2
G.7		24.5 ± 3.2	34.1 ± 2.7		26.8 ± 3.0	30.5 ± 3.6		25.8 ± 2.2	28.5 ± 2.7

Tabla 6. Número de capilares por fibra (NCF) en cada campo para las tres especies de aves salvajes estudiadas: ánade real, focha común y gaviota patiamarilla. Medias expresadas con los límites de confianza al 95%.

	Ánade real			Focha			Gaviota		
	SO	FOG	FG	SO/SW*	FOG	FG	SO	FOG	FG
E.1		3.8 ± 0.3	4.6 ± 0.6	5.7 ± 0.6	5.8 ± 0.8	6.0 ± 0.7	6.4 ± 1.2	5.8 ± 1.1	6.3 ± 1.3
E.2		3.6 ± 0.4	4.1 ± 0.6	5.5 ± 0.7	6.1 ± 0.7	6.2 ± 0.7	5.5 ± 0.9	5.3 ± 1.0	5.8 ± 1.3
T.1		3.4 ± 0.2	4.7 ± 0.5		4.9 ± 0.3	5.3 ± 0.5		5.1 ± 1.0	5.4 ± 1.1
T.2		3.5 ± 0.3	4.8 ± 0.4		5.0 ± 0.8	5.5 ± 0.6		5.1 ± 0.9	5.5 ± 0.6
T.3		3.8 ± 0.4	4.9 ± 0.3		4.9 ± 0.4	5.4 ± 0.4		5.0 ± 0.9	5.6 ± 1.4
P.1		3.3 ± 0.4	4.3 ± 0.4		5.0 ± 0.5			5.0 ± 0.5	
P.2		3.3 ± 0.4	4.5 ± 0.4		4.8 ± 0.6			4.8 ± 0.5	
P.3		3.5 ± 0.4	4.6 ± 0.6		4.6 ± 0.6			4.7 ± 0.6	
P.4		3.6 ± 0.6	4.7 ± 0.6	5.3 ± 0.4*	4.4 ± 0.4			5.1 ± 0.7	
S.1		3.7 ± 0.3	4.6 ± 0.6		5.0 ± 0.3	5.9 ± 0.2		4.9 ± 0.8	5.5 ± 0.9
S.2		3.6 ± 0.3	4.9 ± 0.8		4.9 ± 0.4	5.6 ± 0.5		4.8 ± 0.7	5.6 ± 0.8
S.3	4.6 ± 0.7	4.1 ± 0.3	4.8 ± 0.4	5.5 ± 0.5	4.9 ± 0.5	5.5 ± 0.4	6.0 ± 1.0	5.0 ± 0.7	5.7 ± 1.0
S.4		3.8 ± 0.5	4.7 ± 0.6		4.6 ± 0.4	5.5 ± 0.5		4.5 ± 0.4	5.0 ± 0.3
S.5	4.9 ± 0.6	3.9 ± 0.4	4.7 ± 0.4	5.6 ± 0.4	5.1 ± 0.3	5.6 ± 0.3	5.7 ± 1.2	4.8 ± 0.8	5.3 ± 0.8
S.6		3.3 ± 0.5	4.5 ± 1.0		4.5 ± 0.5	5.3 ± 0.6		4.5 ± 0.3	5.2 ± 0.5
I.1	3.5 ± 0.2	3.5 ± 0.1	4.2 ± 0.5		4.4 ± 0.3	5.0 ± 0.4	4.9 ± 1.1	4.1 ± 0.5	4.4 ± 0.6
I.2		3.5 ± 0.4	4.2 ± 0.6		4.5 ± 0.4	5.1 ± 0.5	5.1 ± 1.1	4.2 ± 0.3	4.5 ± 0.8
I.3	4.8 ± 0.5	4.3 ± 0.4	5.0 ± 0.6	5.5 ± 0.5	4.7 ± 0.5	5.4 ± 0.7	5.4 ± 0.4	4.3 ± 0.3	4.4 ± 0.5
I.4	4.6 ± 0.3	4.8 ± 0.7	4.2 ± 0.3	5.5 ± 0.6	4.7 ± 0.4	5.4 ± 0.6	5.3 ± 0.6	4.2 ± 0.5	4.6 ± 0.5
G.1	5.4 ± 0.5	4.8 ± 0.6	5.5 ± 0.6	5.0 ± 0.8	5.2 ± 0.8	5.9 ± 0.8	5.4 ± 0.6	5.0 ± 0.6	5.1 ± 0.5
G.2	5.9 ± 0.8	5.4 ± 0.6	6.6 ± 0.9	5.2 ± 0.6	5.4 ± 0.7	5.7 ± 0.6	5.1 ± 0.9	5.1 ± 0.7	
G.3	5.9 ± 0.7	5.9 ± 0.9	6.2 ± 1.3	5.6 ± 0.9	5.4 ± 1.0	5.9 ± 0.8	5.4 ± 0.5	5.0 ± 0.6	
G.4	5.0 ± 2.3	4.7 ± 0.7	5.7 ± 1.1		5.2 ± 0.6	5.6 ± 0.6	5.0 ± 0.7	4.7 ± 0.5	4.9 ± 0.8
G.5		4.3 ± 0.5	5.2 ± 1.0		5.1 ± 0.5	5.4 ± 0.5		4.5 ± 0.4	4.9 ± 0.5
G.6		4.4 ± 0.7	5.3 ± 0.8		5.3 ± 0.5	5.3 ± 0.5		4.4 ± 0.4	4.5 ± 0.5
G.7		4.2 ± 0.6	5.3 ± 0.9		5.2 ± 0.5	5.2 ± 0.5		4.6 ± 0.6	4.9 ± 0.5

Discusión

CUESTIONES METODOLÓGICAS

La presente tesis consta de una primera parte de desarrollo metodológico donde se describen dos técnicas de tinción histoquímica. La primera de ellas (**I**), una técnica ATPasa, puede ser empleada para la cuantificación de la red capilar (CD, C/F y NCF) y para la medición de parámetros relacionados con la morfometría fibrilar (FCSA, FPER, MDD, CCA y CCP). La segunda (**II**), que combina el ensayo mATPasa de Brooke & Kaiser (1970) con una técnica AChE, es de gran utilidad para describir aspectos relacionados con la estructura y distribución de los terminales nerviosos, aportando un importante recurso para la clasificación de las fibras musculares.

1. Tinción ATPasa para el contaje capilar

1.1. Justificación metodológica

Desde las primeras observaciones de los capilares en tejidos animales, realizadas por Anton van Leeuwenhoek (1674) en la cola de la anguila, hasta nuestros días se han desarrollado gran número de técnicas histológicas con la finalidad de poner de manifiesto la red capilar del tejido muscular. Las técnicas basadas en la perfusión de los capilares con diferentes sustancias (tinta china y otros colorantes), dan como resultado contajes inferiores a los reales debido a que no todos los capilares están abiertos a la circulación al mismo tiempo (Krogh 1919; Martin *et al.* 1932). Aquéllas que se basan en la tinción de los eritrocitos pueden también infravalorar la CD, dada la elevada probabilidad de que éstos no estén presentes en cortes finos de tejido (Plyley & Groom 1975). Por todo ello, entre los ensayos más recomendables para el contaje de la CD, las técnicas histoquímicas basadas en la localización de la actividad de algún enzima del endotelio capilar (fosfatasa alcalina, amilasa-PAS, ATPasa) son las que han dado mejores resultados, no sólo por su corrección metodológica, sino también por su sencillez.

Si además de obtener valores de CD se desean hacer mediciones de parámetros morfométricos, como el área o el perímetro de las fibras musculares, resulta muy recomendable poseer un método histoquímico que permita mostrar claramente la red capilar y los contornos individuales de cada fibra muscular. La técnica presentada en el artículo **I** de esta tesis consigue este doble propósito mediante la modificación del método ATPasa de Rosenblatt *et al.* (1987).

1.2. Fundamentos técnicos

Meijer publicó en 1970 una técnica ATPasa para la clasificación de las fibras musculares. Esta técnica detectaba la actividad ATPasa a partir de la precipitación del sulfuro de plomo disuelto, junto con el substrato de la reacción (ATP), en el medio de incubación. Rosenblatt *et al.* (1987) modificaron la temperatura de incubación de los cortes (37°C en lugar de 22°C) obteniendo una mejor calidad en la demostración de los capilares, la cual fue atribuida a la termosensibilidad de la ATPasa del endotelio capilar. De hecho, el

método de Rosenblatt *et al.* (1987) conseguía una doble finalidad: poner de manifiesto, en un mismo corte histológico, la red capilar y el tipo de fibra. Aunque estos autores aplicaron con éxito la técnica en la musculatura de algunas especies de mamíferos (resultando no ser útil en muestras de conejos y humanos), en nuestro laboratorio la aplicamos en músculos de aves con resultados no muy alentadores, ya que se hacía difícil identificar los capilares que rodeaban fibras con tinción ATPasa muy elevada. No obstante se observó que, al preincubar en un baño termostatado a 25°C, no sólo desaparecía la tinción ATPasa de las fibras musculares, sino que además se veía incrementada la calidad de tinción de los capilares que se observaban de forma más nítida. Por ello nos propusimos contrastar los contajes capilares obtenidos con ambas técnicas con el fin de valorar la conveniencia de la modificación introducida.

1.3. Validación del método

La preincubación de los cortes a T=25°C mejoró el contaje capilar no sólo en aquellos músculos donde la actividad ATPasa de las fibras musculares resultó ser elevada, lo cual es evidente con sólo observar las microfotografías (**I**: Figs. 1-4), sino también en aquellos músculos compuestos por fibras con tinciones ATPasa bajas o moderadas. Dado que es bien conocido que la CD es altamente dependiente de la FCSA (Ripoll *et al.* 1979), se valoró la posibilidad de que las mayores CD contadas en los cortes preincubados a T=25°C fueran debidas a una disminución del tamaño de las fibras, como resultado de posibles deformaciones en el tejido causadas por la mayor temperatura de preincubación. Dos evidencias permiten descartar esta hipótesis. En primer lugar, la ausencia en todos los músculos muestreados de diferencias estadísticas entre los valores de FCSA y FPER obtenidos a T=4°C y T=25°C (**I**: Tabla 2). En segundo lugar, la presencia de diferencias significativas del mismo orden que en la CD (10%) para el C/F, un parámetro que no se ve influenciado por las dimensiones fibrilares (Egginton & Ross 1992).

Se valoró también la posibilidad de que las características particulares de algún músculo concreto pudieran, de una manera u otra, sesgar los contajes. La homogeneidad de los valores obtenidos para el cociente T4/T25 en todo el rango de CD (**I**: Tabla 1) indica, una vez más, que la infravaloración del contaje capilar es debida únicamente a cuestiones metodológicas.

Los resultados obtenidos ponen de manifiesto que una mayor temperatura de preincubación de los cortes inhibe la actividad ATPasa de las fibras musculares, a la vez que incrementa la actividad ATPasa del endotelio capilar. Aunque no se conoce con certeza cuál es el factor responsable de la actividad ATPasa del endotelio capilar, sí se conocen algunas de sus características, como la resistencia a la preincubación ácida o la activación por diversos cationes (Freiman & Kaplan 1960), a las cuales debe añadirse la termosensibilidad que demuestra el presente estudio.

El rango de variación de CD y C/F, junto con la homogeneidad que presenta el índice T4/T25, permitió calcular una ecuación de regresión que relaciona los resultados obtenidos por ambos métodos (**I**: Figs. 13 y 14). A partir de las fórmulas de estas ecuaciones de regresión es posible interpolar los valores obtenidos con el método de Rosenblatt *et al.* (1987) para T=4°C y obtener los respectivos datos corregidos para T=25°C.

2. Tinción combinada mATPasa-AChE

2.1. Justificación metodológica

Botezat (1909) describió por primera vez dos tipos de terminaciones nerviosas en la musculatura esquelética de aves: un tipo en forma de placas (*"en plaque"*) y otro en forma arracimada (*"en grappe"*). Posteriormente, diversos investigadores mostraron que, tanto en aves (Krüger 1949; Krüger & Günter 1958) como en anfibios (Günter 1949; Couteaux 1952), estos dos tipos de inervación representan dos tipos de uniones neuromusculares que están estrechamente relacionadas con la función de la fibra muscular. A partir de 1970, con la aparición de las técnicas mATPasa de tinción histoquímica (Brooke & Kaiser 1970; Guth & Samaha 1970; Meijer 1970), se pudieron clasificar las fibras musculares en diversos tipos los cuales demostraron presentar diferencias en sus propiedades metabólicas y fisiológicas (Peter *et al.* 1972; Burke *et al.* 1973). A pesar de la importancia que la histoquímica convencional ha adquirido desde entonces, únicamente se ha publicado una técnica de tinción que permite poner de manifiesto, sobre un mismo corte histológico, el tipo de fibra muscular junto con la estructura de su terminal nervioso. Ashmore *et al.* (1978) combinaron una tinción mATPasa con la técnica de Toop (1976) para el revelado de la actividad AChE. No obstante, la nomenclatura utilizada por estos autores para la clasificación de las fibras (α y β) se encuentra fuera de uso, debido a la inusual preincubación ácida que utilizaron. Por ello, se creyó conveniente poner a punto una técnica histoquímica que permitiera clasificar las fibras musculares según la nomenclatura clásica de Brooke & Kaiser (1970), que posteriormente fue adaptada en aves por Barnard *et al.* (1982).

2.2. Fundamentos técnicos

Se plantearon dos cuestiones principales con el fin de mejorar la mencionada técnica de Ashmore *et al.* (1978). En primer lugar, no sólo se consideró la preincubación ácida sino que también se procesaron un grupo de cortes preincubados a pH alcalino. Dado que la actividad mATPasa en las fibras I queda inhibida a un pH elevado, los terminales nerviosos pudieron ser claramente puestos de manifiesto en este grupo de cortes. Este hecho tiene importancia si se considera la posibilidad real de que la unión neuromuscular en las fibras I quede enmascarada a pH ácido, debido a que la mATPasa de estas fibras es muy activa a pH bajos (pH=4-5) y que los terminales nerviosos de las fibras lentas poseen actividades AChE bajas (Buckley & Heaton 1971; Lyles & Barnard 1980).

En segundo lugar, se observó que, tras aplicar la técnica de AChE de Toop (1976), aparecían dispersas en el campo de observación granulaciones negras en forma de precipitado. Se valoró la posibilidad de que estos precipitados fueran debidos a la utilización de elevadas concentraciones (del 20%) de nitrato de plata. Se modificó, por tanto, esta concentración reduciéndola al 5%.

2.3. Validación del método

La primera cuestión importante a considerar fue contrastar los cortes procesados únicamente con la técnica mATPasa con aquéllos procesados según la técnica combinada. La ausencia de diferencias en los tipos de fibras obtenidos mediante ambas técnicas permite concluir que la técnica AChE de Toop (1976) no altera la tinción mATPasa de Brooke & Kaiser (1970).

En segundo lugar, se procesaron cortes musculares de mamífero y de ave con el fin de adecuar las modificaciones metodológicas necesarias para cada grupo de vertebrados. Además de las diferencias encontradas en diversas características del terminal nervioso (**II**: Figs. 1-8; **III**), se observó que en la musculatura lenta de aves se necesita, para demostrarse claramente el terminal nervioso, un tiempo de incubación tres o cuatro veces superior al empleado para los músculos de mamífero. Esta diferencia es debida a la inferior actividad AChE cuantificada en la musculatura lenta de aves (Buckley & Heaton 1971; Lyles & Barnard 1980).

Finalmente se pudo comprobar que la reducción del 20% al 5% en la concentración de nitrato de plata, además de resultar favorable económicamente, mejora la calidad de los cortes ya que los precipitados en forma de granulaciones desaparecieron.

FIBRAS Y PARÁMETROS MUSCULARES

1. Tipos de fibras

Como ya se ha comentado en una sección anterior, a partir de la nomenclatura de Brooke & Kaiser (1970), las fibras musculares se clasifican según su actividad mATPasa. En la musculatura esquelética de mamíferos, el comportamiento estable o lábil de las fibras después de preincubaciones ácidas y alcalinas permite una primera distinción en fibras de tipo I y de tipo II (las cuales se subdividen en IIA y IIB). En aves, además, se añade un tercer tipo de fibra, el tipo III, de acuerdo con Barnard *et al.* (1982).

Se identificaron histoquímicamente todos estos tipos de fibras en el estudio realizado en los músculos de paloma (**III**). La utilización de otros ensayos histoquímicos, además del de Brooke & Kaiser (1970), permitió describir con mayor detalle las características histoquímicas de las fibras musculares en las aves estudiadas. Además, la aplicación de la metodología combinada mATPasa y AChE descrita en el artículo **II**, posibilitó caracterizar, sobre un mismo corte histológico, dos aspectos importantes de la fibra muscular: su perfil mATPasa y las características de su inervación.

1.1. Fibras lentas *(Slow)*

Los resultados obtenidos describen características inéditas que, junto con hallazgos previos, corroboran la presencia de dos tipos de fibras lentas (tipo I y tipo III) en la musculatura esquelética de aves.

1.1.1. Fibras de tipo III o tónicas (slow tonic)

Es bien conocido que las fibras de tipo III poseen un perfil mATPasa estable a pH ácido y alcalino, una estructura del terminal nervioso en forma arracimada (*"en grappe"*) y múltiples terminales nerviosos por fibra (véase la revisión de Morgan & Proske 1984). La presencia de estas fibras se ha detectado, por ejemplo, en el músculo ALD de algunas especies de aves como la paloma (**III**: Tabla 1, Fig. 1A,B,C; Hather & Hikida 1988), el pollo (Asiedu & Shafiq 1972), la codorniz (Alway *et al.* 1990) y el ánade real (**II**: Fig. 8); y en el BRC de paloma (**III**: Fig. 2A,B) y en el del pollo (Nene 1977).

La presente tesis muestra, además, que las fibras III del ALD y del BRC de paloma poseen dos características previamente no descritas de manera cuantitativa:

a) Elevada densidad de inervación, con frecuencias medias (ver metodología artículo **III**) del 14.27% en el ALD y del 14.86% en el BRC (**III**: Tabla 3).

b) Distribución uniforme de los terminales nerviosos, como se deduce de la comparación de las medias y las varianzas de las frecuencias de inervación (**III**: Tabla 6).

Otras características estructurales propias de las fibras III, no investigadas en esta tesis, son la presencia de un sistema sarcotubular poco desarrollado, una menor sensibilidad a la acetilcolina, cadenas pesadas de miosina y propiedades de membrana muy diferentes a las de las fibras de tipo II (Fedde 1969; Hayashi & Nagata 1991; Williams & Dhoot 1992; véase también Vrbová *et al.* 1995). Todo ello refleja las características fisiológicas de estas fibras, las cuales poseen bajas velocidades de contracción, prolongados tiempos de relajación e incapacidad de propagar potenciales de acción (Alexander & Goldspink 1977; Hather & Hikida 1988, Alway 1994; Reiser *et al.* 1996). Así pues, en base a criterios metabólicos y fisiológicos, estas fibras también han sido denominadas fibras **tónicas** (*slow tonic*) y se les ha atribuido un rol postural, por su capacidad en la producción de contracciones prolongadas de bajo consumo energético (Alexander & Goldspink 1977; Meyers 1992).

Tanto el ALD como el BRC de paloma poseen los patrones de inervación y perfiles histoquímicos compatibles con las características de las fibras tónicas. Por ello, y dada la homogeneidad fibrilar de ambos músculos, se puede concluir que están altamente especializados en la realización de funciones posturales que implican contracciones largas y sostenidas. El ALD presumiblemente juega un papel importante en el mantenimiento del húmero pegado al cuerpo mientras que el BRC estaría principalmente implicado en el repliegue del antebrazo sobre el brazo cuando el ave tiene las alas recogidas.

1.1.2. Fibras de tipo I (slow oxidative, SO)

En el artículo **III** se encontraron diferencias histoquímicas entre las fibras de tipo III y las de tipo I. La primera y más destacable es la inversibilidad de la tinción mATPasa a pH ácido y alcalino en las fibras I (**III**: Fig. 1G,H). En segundo lugar cabe señalar la baja actividad SDH observada en las fibras I del SMP. Khan (1979) obtuvo resultados análogos en este mismo músculo denominando a estas fibras *slow glycolytic* o tipo I *white*, debido a su elevada actividad lactato deshidrogenasa. No obstante, con la excepción de las fibras de la zona profunda del PEC de focha (fibras SW) donde también se obtuvieron bajas actividades SDH (**V**: Fig. 5C,D,E), las tinciones histoquímicas de este enzima oscilaron entre moderadas y altas en el resto de músculos (**III**: Fig. 1I; **IV-VI**: Tabla 1). Por esta razón, con estas dos notables excepciones, a las fibras de tipo I se las ha denominado SO (*slow oxidative*). En tercer lugar, la estructura del terminal nervioso posee ligeras diferencias entre ambos tipos de fibras: como ya destacaron Hikida & Bock (1974), en el SMP la unión neuromuscular suele mostrarse como una serie de puntos alineados (*"little knobs"*) en contraposición a la estructura arracimada (*"en grappe"*) de las fibras III. Además de estas características ya conocidas, las fibras I y III de los músculos de la paloma poseen otras diferencias previamente no descritas de manera cuantitativa:

a) Las frecuencias de inervación (véase la metodología del artículo **III**) de las fibras I son en general más bajas que las de las fibras III, mostrando diferencias con una significación de $0.01 < P < 0.05$ (**III**: Tabla 5). En concreto en el SMP se obtuvo una media del 12.73% y en el ITC del 10.04% frente a los 14.27% y 14.86% del ALD y BRC (**III**: Tabla 3).

b) La distribución de los terminales nerviosos en las fibras de tipo I tiende a ser aleatoria en lugar de uniforme, lo cual es especialmente claro en la parte posterior del SMP donde se obtuvieron relaciones media/varianza del orden de 12.28/12.74 frente a las 14.09/4.53 y 15.21/6.10 de ALD y BRC (**III**: Tabla 6).

A pesar de todas estas diferencias entre fibras I y III, la inervación múltiple de ambos tipos de fibras, demostrada en los cortes longitudinales (**III**: Fig. 2A,E), ha suscitado una gran controversia sobre la conveniencia o no de aceptar la presencia de dos tipos de fibras lentas en la musculatura esquelética de aves. Se admite la presencia de fibras tónicas en los músculos de aves y de otros grupos de vertebrados como los anfibios y reptiles (Morgan & Proske 1984). El problema surge cuando se intentan equiparar las fibras I de aves a las fibras I de los mamíferos ya que en este grupo no presentan inervación múltiple, poseen placas motoras (véase artículo **II**) y propiedades contráctiles fásicas (*twitch*) como las encontradas en las fibras II (Morgan & Proske 1984). Por esta razón, algunos autores denominan a las fibras I de aves *"avian slow-twitch oxidative"* (Maier 1983) para diferenciarlas de las fibras I de mamíferos. Aunque no se han realizado estudios electrofisiológicos que permitan establecer las posibles diferencias derivadas de esta dicotomía estructural en la musculatura lenta de aves, un gran número de trabajos asumen la existencia de dos tipos de fibras lentas en aves (véase por ejemplo Hikida 1987; Rosser *et al.* 1987; Meyers 1992). De hecho, la presencia de dos tipos de fibras lentas con propiedades fisiológicas diferentes ha sido descrita en otros grupos de vertebrados como reptiles (Proske & Vaughan 1968) y anfibios (Lännergren 1979).

Las fibras I, que en los trabajos **IV-VII** son denominadas SO, se encuentran en varios músculos locomotores de aves. Exceptuando el caso de la parte posterior del SMP de la paloma, donde su presencia es exclusiva, siempre se encuentran mezcladas en diferentes proporciones entre las fibras rápidas (*fast*). La utilización del ensayo histoquímico GPDH y la tinción Sudan B para lípidos, aportan más información sobre el metabolismo de estas fibras (**IV-VI**: Tabla 1). Las fibras SO no poseen un metabolismo glicolítico, siendo su principal fuente energética el metabolismo lipídico. Las tinciones SDH y Sudan B moderadas en la mayor parte de los músculos presumiblemente indican que el metabolismo energético de estas fibras no es muy elevado en relación con las fibras de tipo II o *fast*. Todas estas características, junto con las particularidades de su inervación, hacen que las fibras SO sean muy adecuadas para desarrollar actividades que requieran una baja tasa de consumo de ATP y que impliquen una fatiga lenta (Alexander & Goldspink 1977). La distribución de estas fibras en las partes más cercanas al hueso y en las zonas profundas de los músculos sugieren una activación mientras las aves se mantienen en pie, en el caso de los músculos de la pata, o complementando la acción de músculos puramente posturales (ALD y BRC), en el caso de los músculos de las extremidades anteriores. En mamíferos se ha demostrado que estas fibras se contraen durante diferentes tipos de ejercicio (Walmsey *et al.* 1978; Armstrong & Laughlin 1985). Por ello, y pese a las grandes diferencias comentadas entre las fibras SO de aves y mamíferos, tampoco es descartable que en aves estas fibras puedan intervenir en algunas funciones locomotoras, realizando movimientos lentos repetitivos o facilitando el almacenamiento de energía elástica en los tendones (Patak & Baldwin 1993).

1.2. Fibras rápidas (Fast)

Las características histoquímicas y metabólicas encontradas en las fibras II de las aves estudiadas son muy diferentes a las de los dos tipos de fibras lentas. En primer lugar, las fibras II presentaron siempre un solo terminal nervioso por fibra con una estructura de verdadera placa motora (*"en plaque"*). La estructura de esta placa motora es equiparable al terminal nervioso de mamíferos aunque, como puede observarse en el artículo **II** (Figs. 5 y 6), en aves se encontraron placas terminales bastante mayores que en la rata. En segundo lugar, los dos subtipos de fibras II presentaron estabilidad a pH alcalino y tuvieron una sensibilidad ligeramente diferente a las preincubaciones ácidas, lo cual permitió clasificarlas en IIA y IIB. A estas características ya conocidas en el PEC de paloma (Koelle & Friedenwald 1949; Chinoy & George 1965; Simpson 1979) cabe añadir los siguientes hallazgos previamente no descritos, los cuales se contemplan como una consecuencia de la presencia de un solo terminal nervioso por fibra:

a) Las fibras II presentaron bajas frecuencias de inervación, las cuales oscilan entre el 1.76% y el 2.92% (**III**: Tabla 3).

b) Se observaron frecuencias de inervación con relaciones media/varianza tan bajas como 2.34/18.14 (en las fibras IIA), que sugieren una distribución agregada de los terminales nerviosos (**III**: Tabla 6, Fig. 2D). Este hecho es compatible con la distribución en mosaico que, en tinciones histoquímicas, presentan las fibras que pertenecen a una misma unidad motora (Vrbová *et al.* 1995) y concuerda con los hallazgos de Trotter *et al.* (1992) en el PEC de codorniz. Estos últimos autores encontraron que el único terminal que las fibras del PEC poseen se distribuye de forma periódica y siempre en la parte central del primer tercio de la fibra. Muy probablemente

la agregación de placas motoras demostrada en esta tesis corresponde a la tinción histoquímica de estas zonas celulares.

c) No se presentaron diferencias significativas entre las frecuencias de inervación de las fibras IIA y IIB ($P > 0.1$), mientras que fueron evidentes grandes diferencias entre las fibras II y los dos tipos de fibras lentas (**III**: Tabla 5).

Como ya ha sido comentado en secciones anteriores, desde un punto de vista metabólico, las fibras II se subdividieron en fibras FOG y FG (**IV-VI**: Tabla 1).

1.2.1. Fibras FG

La baja actividad SDH de las fibras FG indica que el ATP en estas fibras se suministra por vía anaeróbica. Este hecho, junto con la elevada intensidad de tinción GPDH y la baja cantidad de depósitos lipídicos (como reflejan las bajas tinciones de Sudan B), sugiere que la energía requerida durante la contracción muscular se obtiene a partir de la glicólisis anaerobia. Por todo ello, estas fibras se fatigan rápidamente y requieren recuperar su abastecimiento energético una vez ha cesado el ejercicio (Alexander & Goldspink 1977; Butler 1991). Estas características metabólicas capacitan a las fibras FG para ser reclutadas durante cortos períodos de intensa actividad y para desarrollar contracciones de elevada potencia.

1.2.2. Fibras FOG

Las fibras FOG obtienen el ATP a partir del metabolismo oxidativo, como puede deducirse de su elevada intensidad de tinción SDH. Pueden utilizar como substrato energético glúcidos (como demuestran las moderadas y altas actividades GPDH) o substancias de naturaleza lipídica (elevada tinción Sudan B). Concretamente en palomas, Butler *et al.* (1977), Rothe *et al.* (1987) y Bordel & Haase (1993) mostraron evidencias de la utilización de carbohidratos como substratos energéticos durante vuelos cortos y al comienzo de vuelos de larga distancia, mientras que las grasas fueron la principal fuente de abastecimiento energético durante vuelos prolongados. Estas fibras oxidativas son muy resistentes a la fatiga y están aparentemente adaptadas para la realización de movimientos rápidos y repetitivos (Alexander & Goldspink 1977).

Los perfiles metabólicos y las características de inervación de estas fibras, junto con la elevada velocidad de contracción que se les ha descrito en el PEC de paloma (Dial *et al.* 1988), hacen que las fibras rápidas (*fast*) sean las únicas que pueden desarrollar las elevadas frecuencias de contracción necesarias para satisfacer los requerimientos metabólicos y energéticos del vuelo (Goldspink 1981; Rosser & George 1986).

2. Morfometría de las fibras SO, FOG y FG de los músculos locomotores

Las propiedades metabólicas de las fibras musculares encontradas en los músculos locomotores de las tres especies de aves salvajes condicionan las características morfométricas de cada tipo de fibra. A partir de la medias globales de FCSA, FPER, MDD y NCF deben destacarse dos resultados interesantes:

a) Los valores morfométricos siempre fueron significativamente menores en las fibras FOG que en las FG. En las fibras FOG, la elevada tasa de consumo de ATP por vía oxidativa impone un continuo abastecimiento de oxígeno a las mitocondrias durante la contracción muscular. Dado que el paso del oxígeno de los capilares a la fibra muscular se realiza por difusión (Weibel 1984), una corta distancia de difusión (MDD comprendidas entre 16 y 27 μm) hace más eficiente el tránsito de oxígeno de los capilares a las mitocondrias. Además del oxígeno, los substratos energéticos (ácidos grasos o glúcidos) y los productos de desecho del metabolismo, ven también favorecido su abastecimiento y eliminación. Las fibras FG, al obtener el ATP por vía anaeróbica, no ven tan limitado su tamaño (MDD desde 24 hasta 35 μm) por cuestiones de abastecimiento energético ya que la reposición de los substratos no debe realizarse de una manera inmediata sino cuando cesa la actividad. La evidencia de que las fibras con elevadas FCSA son capaces de producir una gran fuerza (Lucas *et al.* 1987; Elzinga *et al.* 1989) se considera un argumento adicional para explicar el gran tamaño de las fibras FG en relación a las FOG. La presumible adaptación de las fibras FG para desarrollar actividades explosivas que requieren del desarrollo de una gran potencia explicaría estas diferencias morfométricas. Por otra parte, las fibras SO poseen un recambio energético más bajo que las fibras FOG, ya que hidrolizan ATP a una velocidad inferior (Goldspink 1981). Esta diferencia en el metabolismo energético de ambos tipos de fibras explica las menores diferencias encontradas entre las fibras SO-FG que entre las fibras FOG-FG (**VII**: Fig. 2 *fibres*).

b) Aunque el NCF a nivel global es similar entre los tres tipos de fibras, en los índices CCA y CCP se observan valores significativamente mayores para las fibras oxidativas (**VII**: Fig. 3 *fibres*). Estos resultados indican que un mayor aumento del flujo de oxígeno a las mitocondrias de la fibra muscular se consigue mediante una reducción de la FCSA y el FPER, en lugar de aumentando el NCF.

3. Variabilidad individual e intramuscular

3.1. Variabilidad individual

La gran variabilidad observada entre individuos de la misma especie, sobretodo en los parámetros CD, FD y C/F (**IV-VI**), plantea la necesidad de considerar minuciosamente el diseño experimental del muestreo antes de realizar estudios sobre la musculatura de animales salvajes. Por otra parte, desde el punto de vista estadístico, al analizar la varianza se deberá considerar el factor *animal o individuo* como bloque. Todo ello es especialmente

preceptivo cuando el tamaño de la muestra es bajo, lo cual ocurre a menudo dada la dificultad de obtener animales salvajes.

3.2. Variabilidad regional

La mayor parte de los músculos estudiados presentaron diferencias zonales para todos los parámetros, siendo espectaculares en algunos casos (por ejemplo en el GLE del ánade real). Esta variación regional debe ser considerada al plantearse cualquier tipo de investigación en la musculatura esquelética. Con la finalidad de no extraer conclusiones erróneas, la obtención de datos sobre parámetros musculares debe implicar un muestreo representativo de toda la sección transversal del músculo. Si, por el contrario, se realizase un muestreo al azar se podrían obtener, dentro de un mismo músculo, valores con rangos de variación del orden del doble en algunos parámetros: en el GLE del ánade real, por ejemplo, se tienen lecturas muy diferentes si se escoge el campo G.5, con CD de 728 capilares · mm^{-2}, o el G.3, con CD de 1385 capilares · mm^{-2} (**IV**: Tabla 2). Este hecho, junto con diferencias metodológicas y la ya comentada variabilidad individual, explica los grandes contrastes en los valores de la CD que existen en la literatura científica. A título de ejemplo, basta comparar los resultados del artículo **II**, que presentan CD entre 1600 y 3100 capilares · mm^{-2} (**II**: Fig. 13), con los obtenidos por Mathieu-Costello (1991) que varían entre 1491 y 5680 capilares · mm^{-2}. Además, si se pretende realizar un estudio comparado entre varias especies de animales, la elección de las diferentes zonas de muestreo debe seguir un diseño común previamente establecido, con el fin de que el propio muestreo no suponga un elemento de distorsión que aumente o anule las diferencias entre especies y regiones musculares.

4. Resultados comparados de la composición fibrilar, capilarización y morfometría muscular

4.1. Morfometría general comparada

Al considerar globalmente la morfometría de un mismo tipo de fibra en cada especie, se observaron grandes similitudes entre las tres aves estudiadas (**VII**: Fig. 2 *birds*). Esto indica que, cuando son consideradas de modo global y a nivel interespecífico, las FCSA, FPER y MDD aparentemente siguen patrones muy fijados en las tres especies de aves estudiadas, con independencia de los hábitos locomotores de cada especie y el parentesco filogenético existente entre ellas. No obstante, cuando se consideraron los índices CCA y CCP, se obtuvieron diferencias interespecíficas significativas, especialmente en las fibras FOG (**VII**: Fig. 3 *birds*). Estas diferencias, detectables sobretodo en el índice CCP, reflejaron valores menores en la gaviota, seguidos de los del ánade real y finalmente de los de la focha (**VII**: Fig. 3c). A partir de estos resultados deben hacerse dos consideraciones importantes:

a) Existe una correlación entre los comportamientos locomotores de cada especie con sus respectivos valores de CCA y CCP. La natación de las gaviotas no es tan activa

como la de patos y fochas (Cramp & Simmons 1985) y, además, las gaviotas poseen un tipo de vuelo que requiere de un menor gasto energético que el vuelo batido desarrollado por patos y fochas (Baudinette & Schmidt-Nielsen 1974; Goldspink *et al.* 1978). Estos comportamientos locomotores reflejan, a nivel global, una presencia de capilares en relación a FCSA y FPER significativamente menor en los músculos de la gaviota que en los músculos de las otras dos especies. Las diferencias significativas que, aunque más pequeñas, se presentan entre el ánade real y la focha podrían ser debidas al hecho de que las fochas realizan pequeños buceos para alimentarse mientras que los ánades reales sumergen únicamente la cabeza en el agua (Del Hoyo *et al.* 1996). La mayor demanda oxidativa que los buceos de corta duración imponen en la fisiología de las aves que poseen estos hábitos alimenticios (Butler 1991) justificaría los mayores valores de CCA y CCP en las fochas que en los ánades reales.

b) La importancia de la relación entre el NCF y el FPER, es decir, del índice CCP. Como resultado del análisis comparado de los valores globales de los índices CCA y CCP se obtuvieron, entre las tres especies, mayores diferencias en el índice CCP que en el CCA (**VII**: Fig. 3b,c). Este resultado subraya la relevancia que tiene la distribución de los capilares por unidad de perímetro fibrilar en el abastecimiento de oxígeno a las mitocondrias de la célula muscular. Tanto Mathieu-Costello (1993), en un estudio comparado de la musculatura de colibrís, atunes y murciélagos, como Snyder (1995) al investigar el crecimiento de la red capilar en aves, llegaron a conclusiones similares sobre la importancia del CCP.

4.2. Músculos implicados en la locomoción aérea

4.2.1. Consideraciones generales

En los músculos de las extremidades anteriores, los dos tipos de fibras oxidativas (SO y FOG) presentaron tamaños (FCSA, FPER y MDD) significativamente mayores en la gaviota que en las otras dos especies de aves estudiadas. El reclutamiento que de estas fibras se realiza durante el vuelo prolongado podría explicar estos resultados. Las gaviotas pasan largos períodos de tiempo con las alas extendidas gracias a las tensiones isométricas que desarrollan los músculos implicados en la locomoción aérea (Goldspink *et al.* 1978). Este tipo de contracciones, como se argumenta más adelante, pueden ser llevadas a cabo de manera más eficaz con FCSA relativamente grandes (1500 a 2500 μm^2). Por el contrario, el elevado número de contracciones que, de manera repetitiva, las fibras FOG de los músculos de los ánades reales y de las fochas deben desarrollar durante el vuelo batido, sólo puede conseguirse con fibras de FCSA pequeñas (750 a 1500 μm^2). Estas fibras FOG, con elevadas demandas metabólicas, ven asegurada una llegada rápida del oxígeno y de los substratos metabólicos a los lugares de consumo celular mediante diámetros pequeños.

Las fibras FG, sin embargo, no presentaron diferencias morfométricas entre los músculos del vuelo. Esto puede ser debido a que en estas aves todas las fibras FG de estos músculos generan contracciones de naturaleza similar, implicadas en la producción de actividades que requieren de una gran potencia. Como se verá en esta sección, los diferentes comportamientos de locomoción aérea que presentan las tres especies de aves (diferentes capacidades de generar vuelo explosivo, por ejemplo) son muy probablemente

el resultado de las diferentes proporciones de fibras FG existentes en los músculos de cada especie, en lugar del reflejo de morfometrías fibrilares distintas.

4.2.2. Consideraciones específicas de cada músculo

Músculo scapulohumeralis caudalis

El SCH de las tres especies consta de una zona profunda donde predominan las fibras oxidativas, se concentran las fibras SO y se encuentran las CD más elevadas del músculo. La presencia de fibras SO sugiere que esta zona puede colaborar en actividades posturales, como el mantenimiento del ala pegada al cuerpo. Las altas proporciones de fibras FOG (80%-90%), abastecidas por CD superiores a 1000 capilares · mm^{-2}, refleja en el caso del ánade y la focha la posible implicación de esta zona también durante el vuelo batido prolongado, en el que el SCH interviene retrayendo, aduciendo y elevando el ala (Dial 1992). En cuanto a la gaviota, la zona profunda del SCH colaboraría, junto con el PEC, en el mantenimiento del húmero extendido durante los largos planeos realizados por estas aves. La mayor presencia de fibras FG (hasta el 45%) y las CD menores (de 700 a 1000 capilares · mm^{-2}) en las zonas superficiales del SCH encajan con el reclutamiento de esta zona durante actividades que requieren desarrollar una gran potencia en un corto período de tiempo. Esto vendría avalado por los registros realizados por Dial (1992) quien, a partir electromiogramas, demostró que el SCH se mantiene especialmente activo durante fases de vuelo a las que denominó fases de vuelo batido discontinuo (*"non-steady flapping flight"*), como por ejemplo aquéllas en las que el ave gana o pierde altura mediante la aceleración o deceleración del batido de sus alas.

A pesar de las mencionadas similitudes en la arquitectura de este músculo, también son muy evidentes diferencias cuantitativas en el porcentaje de fibras, la capilarización y la morfometría de las fibras musculares entre las tres especies de aves. Se observa un mayor grado de regionalización, es decir mayores diferencias entre zonas, en los SCH del ánade real y de la focha que en el SCH de la gaviota (véanse las proporciones de fibras de la Fig. 1 y las CD de la Tabla 2). De hecho el test de comparaciones múltiples entre campos no mostró diferencias significativas en ningún parámetro del SCH de gaviota (**VI**), mientras que distintos niveles de significación sí fueron evidentes en el pato y la focha (**IV**: Fig. 6; **V**: Fig. 7). Los patrones de vuelo de estas especies podrían explicar estas diferencias. El desarrollo del vuelo planeado durante largos períodos de tiempo y el uso ocasional que hacen las gaviotas del vuelo batido discontinuo (Cramp & Simmons 1985) justifican, en estas aves, la existencia de un SCH menos compartimentalizado que en los patos o las fochas.

Músculo triceps scapularis

El TSC del ánade real mostró características generales similares con el de la focha. En ambas especies se observó un gradiente de fibras oxidativas y de capilarización mucho más marcado que en el TSC de gaviota, el cual en esta especie es incluso inexistente para la CD (Tabla 2). Este músculo es utilizado para extender y estabilizar el codo durante la elevación y el descenso del ala (Dial 1992). Esta actividad, si se realiza de manera intensa (como en fases de vuelo batido discontinuo), requerirá importantes proporciones de fibras FG. La distribución de estas fibras en las zonas posteriores del músculo dota de un momento de fuerza mayor a las fibras de esta zona que a las de las zonas anteriores, consiguiéndose así

una mayor eficacia en la estabilización y extensión del antebrazo. La utilización de las fibras FOG, resistentes a la fatiga, se llevaría a cabo durante el vuelo batido prolongado, cuando se requieren contracciones musculares repetidas y continuadas. En el pato y en la focha, el predominio de fibras FOG en las partes anteriores viene acompañado de CD superiores a las de la zona posterior (814 *vs.* 1063 capilares · mm^{-2} en el pato; 1042 *vs.* 1371 capilares · mm^{-2} en la focha), satisfaciendo así los mayores requerimientos de flujo de oxígeno durante el vuelo prolongado.

En la gaviota el menor grado de variaciones regionales entre las zonas anteriores y posteriores del TSC se contempla, como en el caso del SCH, como una consecuencia de la baja maniobrabilidad y de la poca necesidad de vuelo batido discontinuo que requieren estas aves. El TSC de la gaviota presenta, además, otro hecho destacable: las fibras FOG son significativamente mayores que en las otras dos especies de aves estudiadas (Tablas 3 y 4; **VII**: Fig. 6e). Este hecho se ve también reflejado en el dendograma de las fibras FOG, donde se observa una segregación de los TSC de pato y focha, por una lado, y el de gaviota por el otro (**VII**: Fig. 4b). Las aves con marcados patrones de vuelo planeado, como es el caso de la gaviota, utilizan el TSC para estabilizar la articulación del codo (Meyers 1992), acción durante la cual los tendones deben soportar tensiones isométricas continuas y elevadas. Las fibras con mayores FCSA, al poseer más unidades contráctiles en paralelo, son capaces de desarrollar una mayor tensión (Callister *et al.* 1992). Todo ello justificaría las características morfométricas descritas en las fibras FOG del TSC de gaviota. Las estimaciones sobre el bajo coste energético requerido por las contracciones isométricas (Pennycuick 1972, 1975), junto con la fuerte dependencia existente entre la FCSA y la CD (Ripoll *et al.* 1979), podrían explicar que, pese a las mayores proporciones de fibras FOG encontradas en el TSC de gaviota, las CD de este músculo sean entre un 20% y un 30% menores que en el TSC de focha (Tabla 2).

Músculo extensor metacarpi radialis

En el ánade real y en la focha, las diferencias en el porcentaje de fibras oxidativas y en la capilarización entre la parte ventral y dorsal de este músculo, indican una cierta división funcional de los dos compartimentos. Las zonas ventrales serían predominantemente reclutadas durante las fases de vuelo explosivo y las dorsales durante el vuelo sostenido. No obstante, en la focha, ambos compartimentos poseen propiedades marcadamente aeróbicas (CD > 1326 capilares · mm^{-2} y proporciones fibras FOG > 70%), lo cual podría reflejar el importante rol que todo el EMR realiza en la extensión continuada de la mano durante el vuelo batido. Se han descrito mecanismos automáticos durante la extensión del codo que reducen el nivel de trabajo del EMR al extender la mano (Vazquez 1994). El efecto de estos automatismos podría explicar la escasa presencia de fibras FG en este músculo, cuya función en períodos en que se requieren potentes contracciones sería realizada de manera indirecta por las fibras FG de los músculos del brazo (como el TSC).
A diferencia de lo ocurrido en el ánade real y en la focha, el EMR de la gaviota presentó valores prácticamente idénticos en los compartimentos ventral y dorsal. Además, los valores de FCSA y FPER de las fibras FOG fueron superiores a los de las otras dos especies (Tablas 3-4), aunque significativamente mayores sólo a los del pato (**VII**: Fig. 6g). La base de estas diferencias probablemente está en la especialización del EMR de gaviota en el desarrollo de contracciones isométricas para mantener la parte final del ala extendida durante el planeo. Para esta finalidad la presencia de fibras SO, aunque en cantidades bajas (6% al 8%), puede resultar de cierta utilidad.

Músculo pectoralis

En las aves, el PEC es el músculo que posee una masa mayor (Hartman 1961), representando entre el 10% y el 20% del peso total del animal (Norberg 1985). Esta importancia viene justificada por el papel preponderante que este músculo desempeña durante el vuelo, ya que es el mayor responsable del descenso del ala y juega también un papel importante decelerándola durante el movimiento de elevación (Biewener *et al.* 1992; Dial 1992). Así pues, de los músculos implicados en la locomoción aérea, el PEC es el que muestra los contrastes más grandes entre las especies estudiadas, tanto a nivel de arquitectura y composición fibrilar como en la distribución cuantitativa de la red capilar. Todo ello constituye un reflejo estructural de las necesidades fisiológicas que imponen los diferentes patrones de vuelo desarrollados por las palomas, ánades reales, fochas y gaviotas.

a) Tipos de fibras del PEC

La primera gran diferencia entre las especies estudiadas se evidencia en la composición fibrilar de sus PEC. El del ánade real (**IV**) y el de la paloma (**III**) contienen fibras FG en diferentes proporciones, las cuales en las zonas superficiales alcanzan porcentajes de hasta el 40%. Presumiblemente estas fibras juegan un papel importante durante el incremento de potencia que se requiere en períodos de actividad intensa como el despegue o el aterrizaje (Rosser & George 1986; Welsford *et al.* 1991). Por el contrario, ni en el PEC de la gaviota ni en el de la focha se encontraron fibras FG.

En el caso de la gaviota, es probable que el PEC necesite reclutar todas sus fibras para el desarrollo de las tensiones isométricas que mantienen las alas extendidas durante el vuelo planeado. Por esta razón la ausencia de fibras FG, no útiles para este tipo de vuelo, podría ser incluso ventajosa para llevar a cabo de manera efectiva los largos planeos. A pesar de ello, el PEC de gaviota no es totalmente homogéneo ya que alrededor del 20% de las fibras FOG presentan una tinción mATPasa superior al resto y contienen una carga lipídica baja (**V**). En ciertas especies de aves, las funciones atribuidas a las fibras FG son presumiblemente asumidas por un subtipo de fibras FOG con carga oxidativa intermedia (Parker & George 1975; Tobalske 1996). Este hecho, junto con la evidencia de que tipos de fibra con estabilidades mATPasa a pH diferentes poseen propiedades contráctiles diferentes (Pette & Staron 1990; Schiaffino & Reggiani 1994), hace atractiva la hipótesis de que las fibras pobres en lípidos y con altas actividades mATPasa del PEC de gaviota puedan desarrollar funciones análogas a las realizadas por las fibras FG en el PEC de paloma o ánade real.

El PEC de la focha presenta una estructura muy similar a la de la mayor parte de las aves migratorias y pequeños paseriformes (Lundgren & Kiessling 1988; Rosser *et al.* 1994). Las fochas, al igual que los patos buceadores, poseen alas cortas y con áreas pequeñas (Greenewalt 1962). Este hecho resulta probablemente una ventaja para reducir la flotabilidad durante el buceo (Raikow 1973), pero requiere de un batido de alas superior por unidad de tiempo durante la locomoción aérea (Rayner 1987). Se han registrado en las fochas 5.8 batidos por segundo frente a los 5.0 del ánade real o los 2.8 de la gaviota (Meinertzhagen 1955). Por tanto, la presencia de un PEC sin fibras FG podría estar relacionada con el mantenimiento de frecuencias de batido elevadas durante el vuelo.

Ahora bien, la ausencia de fibras FG genera algunos problemas si se requiere de la producción de contracciones de gran potencia. Quizá por esta razón, las fochas son incapaces de realizar despegues verticales y necesitan "correr" bastantes metros por encima del agua antes de obtener el impulso necesario para alzar el vuelo (Rüppell 1977).

La presencia de una pequeña proporción de fibras SW en la parte profunda del PEC de focha podría estar relacionada con la necesidad de mantener las alas pegadas al cuerpo durante los cortos períodos de buceo (10-20 s) que estos animales realizan para alimentarse (Del Hoyo *et al.* 1996). Aunque la presencia de un tipo de fibra lento con baja capacidad oxidativa (SW) no deja de ser sorprendente, este tipo de fibra también ha sido descrito en otros músculos. Sin ir más lejos, las fibras I del SMP de paloma (artículo **III**) poseen las mismas características que las fibras SW del PEC de la focha. Rosser *et al.* (1987) encontraron, en el PEC de codorniz (*Coturnix japonica*), también un tipo de fibra lento y anaeróbico en la misma zona y en las mismas proporciones que las descritas en esta tesis para la focha. En la musculatura lenta tónica de anfibios también se han descrito fibras con muy pocas mitocondrias y bajas reservas energéticas (Lännergren & Smith 1966; Engel & Irwin 1967; Smith & Ovalle 1973; Mutungi 1990). Pese a estas características metabólicas, se ha argumentado que, debido a la baja velocidad de contracción que caracteriza a estas fibras, el mantenimiento de la tensión debe ser mucho más económico y, por tanto, compatible con pocas cantidades de mitocondrias (Lännergren 1975).

b) Morfometría de las fibras y capacidad oxidativa del PEC

Los valores morfométricos (FCSA, FPER y MDD) presentaron marcadas diferencias significativas entre las fibras del PEC de las tres especies (**VII**: Fig. 6h). Las fibras FOG del PEC de la gaviota siempre tuvieron valores de FCSA que superaron al menos en un 50% a los de las fibras FOG del PEC de ánade real y de la focha (Tabla 3), traduciéndose en mayores FPER y MDD (Tablas 4 y 5). Además las CD de las zonas con predominio de fibras FOG fueron inferiores (entre un 10% y un 30%) en la gaviota, así como también la actividad SDH, reflejada en la intensidad de la tinción, que fue moderada en la gaviota (**VI**: Fig. 5G) frente a las intensas coloraciones presentes en la paloma (**III**: Fig. 5F), ánade real (**IV**: Fig. 5F) y la focha (**V**: Fig. 5C). Dos factores podrían explicar estos contrastes:

1. El tipo de contracción de las fibras musculares. Como ya ha sido comentado en el caso del músculo TSC, las fibras con tamaños grandes son capaces de desarrollar tensiones elevadas que serían de gran utilidad para mantener las alas extendidas incluso en condiciones atmosféricas adversas. Además, dado que se ha descrito que la actividad incrementa y la inactividad reduce la FCSA de una fibra (Nicks *et al.* 1989; López-Rivero *et al.* 1992*b*), la selección de tamaños de fibra del orden de los obtenidos en el PEC de gaviota (1500 μm^2) puede haber sido un factor complementario para optimizar la estructura muscular de estas aves a las características del vuelo planeado, el cual requiere de un constante uso de las fibras FOG durante largos períodos de tiempo.

2. La demanda energética del tipo de vuelo. La tasa de consumo energético en el vuelo batido es una de las más elevadas de todos los modos de locomoción (Rayner 1987). La mayor parte de la energía requerida para el vuelo batido se obtiene a partir de la contracción continuada y repetida del músculo PEC (Pennycuick 1989). Sin embargo, en el vuelo planeado la demanda energética es muy inferior, ya que el PEC sólo es el responsable del mantenimiento de las alas extendidas, pues la mayor parte de la energía

se obtiene a partir de los movimientos de la atmósfera (Norberg 1985; Pennycuick 1989). Estas mayores demandas energéticas no sólo imponen una mayor actividad muscular (Goldspink *et al.* 1978) sino también unos valores superiores de consumo de oxígeno (Baudinette & Schmidt-Nielsen 1974) y frecuencia cardíaca (Butler & Woakes 1980), mayores volúmenes sanguíneos (Palomeque & Planas 1978; Viscor *et al.* 1985) y hematocritos y contenidos en hemoglobina (Pagès & Planas 1983; Viscor *et al.* 1984) más elevados en las especies que desarrollan vuelo batido. Del presente estudio se deduce que a todo ello debe añadirse, como un medio para hacer más eficaz la llegada de oxígeno a las mitocondrias, la presencia de fibras oxidativas con FCSA pequeñas (de 700 a 1000 μm^2); cortas MDD (de 16 a 20 μm); altos valores de CD, que oscilan en patos y fochas de 1300 a 1600 capilares · mm^{-2} (Tabla 2) y que en palomas alcanzan valores medios de 2362 capilares · mm^{-2} (**I**: Tabla 1); y grandes cantidades de mitocondrias, como se deduce de la elevada tinción SDH.

4.3. Músculos implicados en la locomoción terrestre y acuática

4.3.1. Consideraciones generales

A nivel general, en los dos músculos de las extremidades inferiores deben destacarse dos resultados.

a) Desde un punto de vista morfométrico, en las fibras de los músculos de las patas se observó una tendencia totalmente opuesta a la encontrada en los músculos implicados en la locomoción aérea. En esta ocasión, no se observaron diferencias significativas en el tamaño de las fibras oxidativas (SO y FOG), mientras que sí se pusieron de manifiesto estas diferencias entre las fibras FG. Este hecho probablemente indica que las fibras oxidativas de las patas llevan a cabo contracciones de la misma naturaleza en las tres aves estudiadas. En el caso de las fibras SO, realizando un importante papel postural mientras el ave se mantiene en pie o actuando como estabilizadoras de las tensiones producidas por las fibras FG y FOG (Suzuki *et al.* 1985). En el caso de las fibras FOG, debe alcanzarse un compromiso entre la capacidad de realizar contracciones que requieren un metabolismo aeróbico y una potencia suficiente para vencer la viscosidad y resistencia al avance que ofrece del medio acuático. Los estrechos márgenes de variación en la FCSA de las fibras FOG (de 1100 a 1300 μm^2 en el ITC y de 1600 a 1800 μm^2 en el GLE) indican un alto grado de optimización como consecuencia de las similitudes en la natación sostenida de estas especies. Por el contrario, en los patos y en las fochas las actividades que requieren de la producción de grandes cantidades de fuerza por unidad de tiempo (*sprint* acuático, por ejemplo) se dan con mucha más frecuencia que en las gaviotas. Este tipo de natación tiene probablemente, en ambas especies, un significado adaptativo mayor que en las gaviotas ya que, por ejemplo, en ánades reales inmaduros que todavía no son capaces de volar el *sprint* acuático es un importante recurso para escapar de los depredadores (Ageldinger & Fish 1995). De hecho, estos autores describen en ánades reales un modo de locomoción acuática que denominan *hydroplaning* el cual, para llevarse a cabo, necesita desarrollar una gran potencia propulsora con las patas. Los resultados que aquí se presentan sugieren que las fibras FG del GLE de fochas y patos, dada su gran FCSA (2500 a 3100 μm^2), son muy adecuadas para poder realizar este tipo de actividades

natatorias. En la gaviota, la presencia de fibras FG significativamente menores (2000 a 2400 μm^2) reflejaría la menor capacidad de natación explosiva que las patas de esta especie son capaces de desarrollar.

b) Los mayores tamaños de las fibras FOG del GLE con respecto al ITC observados en las tres especies de aves son presumiblemente una consecuencia de la mayor implicación que el GLE tiene en la locomoción, especialmente durante la natación. El movimiento cíclico de la pata durante la natación consta de dos fases (Clark & Fish 1994). Una de propulsión, durante la cual las membranas interdigitales de patos y gaviotas o las expansiones lobuladas de las fochas están totalmente extendidas, y una fase de recuperación, cuando el fémur se retrae y se repliegan estas membranas para disminuir las fuerzas de rozamiento con el agua. Como el GLE es el responsable de la extensión del tarsometatarso y el ITC se encarga de retraer el fémur (Cracraft 1971), el GLE debe ejercer una mayor fuerza para vencer la resistencia del agua durante la propulsión que la requerida por el ITC durante la fase de recuperación. Otra evidencia que demuestra la mayor implicación del GLE que el ITC en la locomoción la constituyen los hallazgos de Sigmund (1959). Este autor describió en las fochas, tanto en la locomoción acuática como en la terrestre, un mayor ángulo de movilidad en la articulación del tobillo que en la de la rodilla.

4.3.2. Consideraciones específicas de cada músculo

Músculo gastrocnemius lateralis (pars externa)

Dado que las tres especies estudiadas son, en mayor o menor medida, aves acuáticas, no debe sorprender que la arquitectura de sus GLE sea muy similar. En las tres especies se encontraron diferencias significativas entre los campos del GLE, lo cual indica claras variaciones regionales en los parámetros oxidativos. La parte anterior del GLE tiene una componente claramente oxidativa, con porcentajes de fibras FOG superiores al 60% y CD que oscilan entre 1000 y 1300 capilares · mm^{-2}. Esta zona es reclutada durante períodos de actividad sostenida (por ejemplo, la natación continuada) como demostraron Butler *et al.* (1988) al encontrar un mayor aumento del flujo sanguíneo en la parte oxidativa que en la parte anaeróbica del GLE del porrón moñudo (*Aythya fuligula*). Además, esta zona muscular posee porcentajes de fibras SO con valores que oscilan, en la parte más cercana al hueso, entre el 20% y el 30%. Estas fibras podrían intervenir en la locomoción terrestre (Walmsey *et al.* 1978; Armstrong & Laughlin 1985), aunque probablemente tengan una mayor importancia en actividades relacionadas con el mantenimiento de la postura (Suzuki & Tamate 1979; Suzuki *et al.* 1982). En claro contraste con la zona anterior, la posterior posee porcentajes de fibras FG que oscilan entre el 40% y el 60%, carece de fibras SO y posee CD de alrededor de 800 capilares · mm^{-2}. Estas características parecen indicar que esta zona del GLE es reclutada durante actividades que requieren del desarrollo de una gran potencia, como cortos *sprints* acuáticos o terrestres.

A pesar de todas estas similitudes, se deben destacar diferencias cuantitativas entre las tres especies, las cuales pueden ser atribuidas a sus diferentes patrones de locomoción acuática y terrestre.

a) Niveles de variación regional

De los tests Scheffé de comparaciones múltiples (**IV** y **VI**: Fig. 7; **V**: Fig. 8) se deduce que el GLE de la gaviota presenta un menor grado de variación regional que las otras dos especies, donde se observan contrastes mucho mayores entre las diferentes zonas del músculo. La heterogeneidad muscular es de gran importancia funcional para graduar y controlar la fuerza muscular durante el movimiento (Vrbová *et al.* 1995). Se considera que los músculos con distribuciones heterogéneas de tipos de fibras, capaces de reclutarlas de forma gradual, pueden desarrollar movimientos más variados (Parkhouse 1988; Hermanson *et al.* 1993). Por ello, las mayores variaciones regionales encontradas en el ánade real y en la focha son contempladas como un reflejo de la mayor variabilidad en las actividades locomotoras que estas aves realizan con las extremidades inferiores. Ambas especies, aunque pueden alimentarse en tierra, principalmente lo hacen en el agua donde necesitan nadar continuamente para obtener materia vegetal y pequeños invertebrados (Del Hoyo *et al.* 1992, 1996). Las fochas, además, realizan cortos buceos, que pueden llegar a alcanzar profundidades medias de 1 a 2,5 m y duraciones de 20 s (Bakker & Fordham 1993; Del Hoyo *et al.* 1996), durante los cuales se propulsan exclusivamente con las patas para la natación subacuática (Neu 1931). Ambas especies mudan sus plumajes de forma masiva, por lo que durante varias semanas no pueden volar (Del Hoyo *et al.* 1992), y dependen totalmente de sus extremidades inferiores para los desplazamientos acuáticos o terrestres. Ánades reales y fochas deben utilizar también las patas para acceder a sus nidos, ya que los de los ánades se ubican en el suelo, entre la vegetación o cavidades naturales (Del Hoyo *et al.* 1992); y los de las fochas en manchas de vegetación emergente en aguas someras (Del Hoyo *et al.* 1996). Al alzar el vuelo desde el agua, los ánades reales realizan un despegue vertical obteniendo impulso adicional a partir de una patada vigorosa con ambas patas (Del Hoyo *et al.* 1992), para la cual una considerable presencia de fibras FG en la parte posterior del GLE puede ser crucial. En el caso de las fochas, durante el despegue y aterrizaje, las fibras FG de las patas deben suplir la ausencia de fibras FG del PEC, realizando *sprints* sobre el agua con la finalidad de impulsarse, antes de alzar el vuelo, o para frenar, al posarse en el agua (Rüppell 1977). Las gaviotas, por el contrario, no hacen un uso tan amplio de sus extremidades inferiores. Cuando no vuelan, lo cual ocupa una gran parte de su tiempo, suelen encontrarse reposando en tierra o boyando en la superficie del agua (Cramp & Simmons 1985). La presencia de grandes cantidades de fibras SO en el GLE demuestra que las patas de estos animales están bien adaptadas para funciones posturales. Aunque son buenas nadadoras, las gaviotas generalmente permanecen sobre la superficie del agua para reposar con una natación menos vigorosa que la desarrollada por patos y fochas (Cramp & Simmons 1985). La presencia exclusiva de fibras oxidativas, con CD alrededor de 1000 capilares · mm^{-2} en las zonas anteriores del GLE y los valores de FCSA significativamente menores respecto a patos y fochas en las fibras FG de todo el GLE (**VII**: Fig. 5b; Tabla 3), podrían ser un reflejo de estos comportamientos nadadores. Las gaviotas, además, mudan el plumaje de manera gradual, empleando para ello cuatro o cinco meses, sin perder la capacidad de volar en ningún momento (Grant 1986), y sus nidos se ubican en zonas costeras, como acantilados o playas, directamente accesibles desde el aire (Cramp & Simmons 1985).

b) Morfometría de las fibras oxidativas

Las fibras FOG del GLE presentaron los mayores valores de FCSA encontrados en los seis músculos estudiados (Tabla 3). Esto es especialmente destacable en el ánade real y en

la focha donde, en algunos casos, la diferencia de tamaño en relación a otros músculos fue hasta del 100% (compárense las FCSA de las fibras FOG del PEC y GLE del ánade real, Tabla 3). Este hecho indica que, en las fibras musculares, elevadas tasas de metabolismo oxidativo pueden ser compatibles tanto con tamaños pequeños de fibra (caso del PEC), como con tamaños relativamente grandes (como en el GLE). En el caso del ánade real, se pueden intuir más claramente estas dos estrategias a partir de la Figura 5 del artículo **IV**. En ella puede observarse claramente que las mayores CD (en GLE y PEC) se corresponden con valores de densidades de fibras muy diferentes. Los mayores valores de FCSA de las fibras FOG del GLE podrían ser una solución adecuada para generar potentes contracciones musculares y vencer de manera eficaz la gran resistencia que el medio acuático ofrece al movimiento. Las mayores MDD (Tabla 5) que presentan las fibras FOG del GLE pueden verse minimizadas por varios factores (Londraville & Sidell 1990). Entre ellos la distribución preferente de las mitocondrias en posiciones subsarcolemales (Weibel 1984) y el gradiente radial de actividad SDH encontrado en las fibras musculares de patos y ocas (Swatland 1984, 1985), juegan seguramente un papel importante. Todos estos hallazgos hacen que el FPER sea un factor relevante al considerar la capacidad oxidativa de la fibra, como ya se ha apuntado con anterioridad en esta tesis.

Músculo iliotibialis cranialis

Se observaron diferencias considerables entre el ITC de gaviota y el de las otras dos especies. El ITC de gaviota resultó ser muy homogéneo, con proporciones de fibras y CD similares entre los cuatro campos estudiados. Por el contrario, en el ánade real y en la focha, el ITC presentó variaciones regionales considerables, con una zona posterior claramente oxidativa y otra anterior con características anaeróbicas. Este patrón de regionalización, inverso al del GLE, es coherente con las posiciones anatómicas respecto al hueso de ambos músculos: la parte posterior del ITC es la zona más cercana al fémur mientras que en el GLE la parte más próxima a la tibia es la anterior. Aunque el principal músculo implicado en la natación es el GLE (Sigmund 1959), el ITC juega probablemente un papel importante durante la fase de recuperación de la patada propulsora. Por tanto, la implicación activa del ITC en la natación y en la retracción del fémur durante la locomoción terrestre (Cracraft 1971) podría explicar, con los mismos argumentos comentados en el caso del GLE, las diferencias de regionalización entre las gaviotas y las otras dos especies de aves estudiadas.

La amplia distribución (15% al 26%) de las fibras SO en las zonas posteriores del ITC sugiere, una vez más, un importante papel postural de estas fibras dada su proximidad al hueso. La presencia de fibras SO en todos los campos estudiados del ITC de gaviota constituye, junto con la amplia distribución de estas fibras en el GLE, una evidencia más que justifica la buena adaptación de la musculatura de las extremidades inferiores de estas aves para desarrollar actividades posturales.

4.4. Arquitectura muscular e implicaciones funcionales

4.4.1 Comparación de los gráficos que relacionan los valores de FD y CD

En la gaviota, la concentración de puntos en una reducida zona del gráfico y la presencia de rectas de regresión que carecen de un patrón común (**VI**: Fig. 6), indica pocas variaciones en la relación entre la FD y la CD en todos los campos de los músculos estudiados. Esta homogeneidad se interpreta como una consecuencia más del bajo nivel de regionalización que los músculos de estas aves presentan. Como consecuencia de la especialización funcional de la mayoría de sus músculos, el gráfico de la gaviota contrasta con la mayor dispersión de puntos observada en los gráficos del ánade real y la focha (**IV**: Fig. 5; **V**: Fig. 6). En ambas especies, la heterogeneidad de la relación FD *vs.* CD se debe a las mayores variaciones regionales mostradas por los músculos del ánade real y de la focha para responder a la mayor cantidad de actividades locomotoras. En los músculos de la focha, la presencia de rectas de regresión con pendientes muy similares y solapadas demuestra una relación lineal entre la CD y el tamaño de las fibras. Este hecho indica que, en los músculos de estas aves, el abastecimiento de oxígeno y substratos energéticos se realiza mediante un perfecto ajuste entre la vascularización y la morfometría de las fibras. Las rígidas restricciones aeróbicas que el vuelo batido y el buceo imponen sobre la fisiología de las fochas podrían ser las responsables de estos resultados. Por el contrario, en el ánade real la dependencia entre FD y CD varió según el músculo estudiado, obteniéndose rectas de regresión con pendientes muy desiguales (**IV**: Fig. 5). Tales relaciones hacen compatible la presencia de una elevada vascularización tanto con fibras con FCSA pequeñas como con FCSA relativamente grandes. Como ya ha sido comentado en la sección anterior, la obtención de altas demandas oxidativas puede conseguirse mediante dos estrategias que se contemplan como un resultado de las diferentes necesidades locomotoras del vuelo batido y de la natación sostenida de superficie.

4.4.2. Análisis de las afinidades entre los diferentes campos musculares

Los tres dendogramas mostrados en los artículos **IV-VI** (Figs. 8 y 9) ponen de manifiesto la importancia funcional de la regionalización muscular. En las tres especies estudiadas, se observa una clara segregación entre los campos musculares donde predominan las fibras FG, con registros bajos de CD, y aquéllos que presentan claras características oxidativas, como altos porcentajes de fibras oxidativas y elevadas CD. Además, también en las tres especies, se observó una separación dentro de los campos oxidativos entre los que poseen fibras SO-FOG y aquellos cuyas fibras oxidativas fueron exclusivamente FOG. Regiones diferentes de músculos capaces de desarrollar actividades muy variadas (como por ejemplo el GLE) se presentan a menudo en grupos totalmente segregados, demostrando así una gran división funcional entre zonas del mismo músculo. Por el contrario, la presencia de campos agrupados con distancias de segregación muy pequeñas, por ejemplo los músculos de ala (EMR y TSC) de la gaviota, no sólo demuestran la analogía funcional entre campos de diferentes músculos sino que también reflejan la elevada especialización funcional de algunos músculos en el desarrollo de actividades muy concretas como, en el caso del EMR y el TSC de la gaviota, el mantenimiento de determinadas partes del ala extendidas durante el vuelo.

Conclusiones

1. En cortes histológicos de músculo, procesados para mostrar la actividad ATPasa, las mayores densidades capilares observadas después de preincubar a T=25°C no son debidas a deformaciones tisulares, causadas por la temperatura, sino a la termosensibilidad de la ATPasa del endotelio capilar. Esta modificación metodológica permite una mayor precisión en la valoración de la densidad capilar que la obtenida por otros métodos similares.

2. El ensayo mATPasa de Brooke & Kaiser (1970) no se ve alterado por la aplicación subsiguiente de un método para la demostración de la actividad acetilcolinesterasa. Ello permite poner de manifiesto, mediante una técnica combinada, el tipo de fibra muscular y su inervación. En aves, son necesarios tiempos de incubación prolongados para mostrar los terminales nerviosos de las fibras lentas debido a su baja actividad acetilcolinesterasa.

3. La musculatura esquelética de aves presenta dos tipos de fibras lentas que, pese a tener características comunes como la inervación múltiple, poseen perfiles histoquímicos y patrones de distribución diferentes. Las características metabólicas de las fibras de tipo III, exclusivas en músculos no implicados en la locomoción como el anterior latissimus dorsi y el brachialis, las hacen óptimas para desarrollar únicamente actividades posturales. Sin embargo, las fibras de tipo I o SO, aunque generalmente puedan estar implicadas en actividades posturales, su distribución (básicamente en la musculatura motora) y sus características no descartan una función locomotora.

4. Los dos subtipos de fibras rápidas o de tipo II, las FOG y las FG, presentan idénticas características de inervación y se diferencian en sus perfiles histoquímicos. Dadas sus características metabólicas, las fibras FOG probablemente se reclutan durante períodos locomotores de actividad prolongada. Sus características morfométricas, como tamaños y distancias de difusión pequeños, permiten un abastecimiento de oxígeno eficiente. Las fibras FG únicamente dependen del metabolismo glicolítico y anaeróbico. Sus grandes tamaños las hacen óptimas para el desarrollo de potentes contracciones durante actividades motoras intensas.

5. Las fibras musculares ven incrementado el flujo de oxígeno a las mitocondrias a través de la reducción del área y el perímetro celular, en lugar de por el aumento del número de capilares que las rodean. Además, se obtuvieron evidencias que insinúan que la distribución de los capilares por unidad de perímetro tiene gran importancia en el aporte de oxígeno a la fibra muscular. Este hecho podría explicar la presencia de una elevada vascularización en músculos con fibras FOG relativamente grandes, restando importancia a la distribución de los capilares por unidad de área.

6. Dada la variabilidad entre individuos de la misma especie, desde un punto de vista estadístico, los estudios en la musculatura de aves salvajes deben realizarse bajo diseños balanceados y considerando el factor *individuo* como bloque en el análisis de la varianza.

7. Las variaciones estadísticamente significativas obtenidas entre zonas del mismo músculo en parámetros como la capilarización, los porcentajes de tipos de fibras y la morfometría muscular imponen, a nivel metodológico, la necesidad de obtener muestreos precisos y representativos de toda la sección transversal del músculo.

8. Los ánades reales y fochas consiguen abastecer las mayores demandas oxidativas impuestas por el vuelo batido con fibras FOG que poseen una elevada actividad oxidativa, reducidas distancias de difusión y altas densidades capilares. Sin embargo, las contracciones isométricas desarrolladas por las gaviotas durante el vuelo planeado se llevan a cabo por fibras con moderada capacidad oxidativa (caso del pectoral) y tamaños significativamente mayores.

9. En las tres especies se observaron fibras FOG mayores en el gastrocnemio que en el iliotibialis. Las características biomecánicas de ambos músculos explicarían este hecho ya que la articulación de la rodilla, donde actúa el iliotibialis, presenta un menor ángulo de movilidad que la articulación del tobillo, donde actúa el gastrocnemio. Además, las tensiones que debe desarrollar el gastrocnemio para vencer la resistencia que el medio acuático opone al avance durante la fase de propulsión son mayores que las que debe realizar el iliotibialis en la fase de recuperación. Para ello las fibras de tamaño relativamente grande deben jugar un papel primordial.

10. Los mayores grados de regionalización presentes en los músculos de ánades y fochas se correlacionan con la gran variedad de actividades locomotoras que estas especies realizan en comparación con las gaviotas:

(a) La capacidad de desarrollar vuelo explosivo en fochas y ánades, con vigorosos batidos de las alas que capacitan la maniobra en espacios reducidos, se refleja en sus músculos triceps, scapulohumeralis y extensor metacarpi radialis que poseen proporciones de fibras anaeróbicas más altas que en los mismos músculos de la gaviota.

(b) La gran potencia requerida durante el despegue y el aterrizaje vertical es proporcionada, en el ánade real y la paloma, por la importante cantidad de fibras FG que poseen estos animales en algunas zonas de su pectoral. Para este tipo de actividades, las fochas, con pectorales exclusivamente formados de fibras FOG, necesitan utilizar las fibras FG de las patas y de los demás músculos del ala para impulsarse; mientras que las gaviotas podrían reclutar las fibras FOG, pobres en lípidos y con intensa tinción mATPasa, que poseen en su pectoral.

(c) Dadas sus características marcadamente oxidativas, la zona anterior del músculo gastrocnemio y la posterior del iliotibialis son presumiblemente reclutadas durante el ejercicio sostenido y en actividades posturales. La mayor proporción de fibras SO en el gastrocnemio de gaviota refleja la buena adaptación de este músculo para el desarrollo de actividades posturales en estas aves. Por el contrario, la zona posterior del gastrocnemio y, en ánades y fochas, la anterior del iliotibialis son claramente anaeróbicas y probablemente más activas durante períodos de intensa actividad (*sprints* acuáticos o terrestres). La mayor frecuencia con que ánades reales y fochas desarrollan este tipo de actividades, y el limitado uso que de estos patrones de locomoción hacen las gaviotas, explicarían los tamaños significativamente mayores de las fibras FG que ánades y fochas poseen en estas zonas.

11. Dependiendo de los requerimientos locomotores de la especie de ave y como resultado de las interacciones entre ésta y su medio ambiente, la musculatura esquelética presenta varios diseños metabólicos y estructurales básicos que ofrecen infinidad de combinaciones para la organización y control del movimiento por parte del sistema nervioso central.

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I

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Original Article

A Histochemical ATPase Method for the Demonstration of the Muscle Capillary Network[1]

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A histochemical method for demonstration of the capillaries in skeletal muscle of birds is proposed. The present method, which is a modification of a previously reported myosin ATPase technique used for simultaneous staining of capillaries and fiber types, provides an accurate count of capillaries associated with different fiber types in avian skeletal muscles. We have applied the original and the modified method to serial adjacent sections of certain skeletal muscles and our results show that after the application of the original technique: (a) in muscles having dark Type II fibers, these fibers produce a masking effect on their adjacent capillaries; (b) a consistent and significant undercounting in capillary densities can be seen even in muscles having no dark Type II fibers; and (c) the staining quality and capillary count are substantially improved with the use of the modified method. We attribute the better results obtained with our modification to differences in thermosensitivity of ATPase activity from the capillary endothelium and of the myofibers. A mathematical treatment is therefore proposed to correct the values of capillary count obtained with the original method. (*J Histochem Cytochem 41:283–289, 1993*)

KEY WORDS: Bird skeletal muscle; Capillary density; Histochemical method.

Introduction

Since Krogh's pioneering work in 1919, several techniques attempting to demonstrate skeletal muscle capillaries have been published. Some of them reveal the capillaries after perfusion of the vascular bed with different substances: India ink (Gray et al., 1983; George and Naik, 1960; Valdivia, 1958; Krogh, 1919), toluidine blue (Mutungi, 1990), a fluorescent dye (Hargreaves et al., 1990; Vetterlein and Schmidt, 1983), or a silicone elastomer of very low viscosity (Plyley and Groom, 1975). However, not all vessels are revealed by these different substances because, as is well known, not all capillaries are uniformly open at the same time. Other techniques are based on the staining of the erythrocytes in the capillary lumen (Heroux and St. Pierre, 1957) or of the capillary walls (Banchero, 1975; Romanul, 1965). Methods involving the staining of red blood cells have the additional shortcoming that the thin slices of muscle tissue might include capillaries that do not contain erythrocytes (Plyley and Groom, 1975), resulting in an undercount of the total number of capillaries.

Among the methods designed to avoid these difficulties, the perfusion-fixation technique (Mathieu–Costello, 1987,1991) and classical histochemical methods, based on the enzyme characteristics of the capillary endothelial cells, have been preferentially used. These involve alkaline phosphatase (Bennett et al., 1991; Gomori, 1939), amylase–PAS (Lundgren and Kiessling, 1988; Andersen, 1975), the immunoperoxidase reaction for *Ulex europaeus* agglutinin (Paljärvi and Naukkarinen, 1990), several ATPase methods (Snyder, 1988,1990; Khan, 1979; Sillau and Banchero, 1977; Meijer, 1970), and a combined ATPase and amylase–PAS method recently described by Hather et al. (1991).

Rosenblatt et al. (1987) described an ATPase technique in which they simultaneously demonstrated the capillaries and the myosin ATPase (m-ATPase) activity of muscle fibers in skeletal muscles of a variety of mammals. They found a 100% fiber-to-fiber correspondence for Type II fibers between the alkaline pre-incubation method of Brooke and Kaiser (1969,1970) and their ATPase technique. They also demonstrated no significant differences in some capillary indexes comparing their technique with the amylase–PAS method.

We have applied the method of Rosenblatt et al. (1987) to several bird muscles (Viscor et al., 1991,1992) and have found that it is difficult to evaluate the exact capillary number in muscles with a high number of positive m-ATPase fibers (Type II according to Brooke and Kaiser, 1969,1970) due to a masking effect produced by these very dark fibers over their adjacent capillaries.

In an attempt to avoid this effect, the present study introduces a modification of the method of Rosenblatt et al. (1987) that shows all the capillaries in contact with each fiber (either Type I or Type II) clearly. In addition, we have found, at least in avian skeletal

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muscles, that the original method considerably undercounts the total capillary number. This difference, due to variations caused by pre-incubation temperature, may be related to the optimal temperature of the endothelial ATPase enzyme activity. A mathematical factor is derived for the correction of the values obtained with the original technique of Rosenblatt et al. (1987).

Materials and Methods

Skeletal muscle samples from two adult domestic pigeons were used for this study. Animals were anesthesized with sodium pentobarbital (75 mg/kg body weight) and M. pectoralis, M. triceps scapulohumeralis, M. pronator superficialis, M. gastrocnemius lateralis, and M. extensor metacarpi radialis [according to Vanden Berge (1979) nomenclature] were dissected out. The samples were quickly frozen in isopentane pre-cooled with liquid nitrogen (Dubowitz and Brooke, 1973) and stored at −160°C in liquid nitrogen until processing. Serial sections of each muscle (14 μm thick) were obtained in a cryostat (Frigocut; Reichert–Jung, Heidelberg, Germany) at −24°C. Sections were mounted on gelatinized slides and processed in two sets: the first was processed according to the original method of Rosenblatt et al. (1987) using the buffered fixative described by Guth and Samaha (1970), whereas the second set was processed with the following modification. The fixation temperature (the first step of the Rosenblatt technique) was 25°C instead of 4°C. This higher fixation temperature inhibited the m-ATPase activity, avoiding the masking effect mentioned above. However, the capillary ATPase activity was apparently not inhibited at this temperature. To check this, serially adjacent sections were fixed at 4°C and 25°C, respectively and, after the same fixation period (5 min), the sections were placed in the same incubation medium (at 40°C, which is physiological temperature for birds), rinsed and stained together, and finally mounted in a glycerol droplet. The M. pectoralis, M. gastrocnemius, and M. triceps sections were used for this purpose because the Type II fibers in these muscles have only slight stain, and thus it is possible to count all the capillaries around each fiber even after fixation at 4°C. To obtain an appropriate number of values for a correct statistical treatment, right and left muscles from each pigeon were processed. These muscles were sectioned at two levels, 1 mm apart, and at each level two fields were randomly chosen to count the capillary (CD) and fiber densities (FD), expressed as capillaries and muscle fibers per mm^2 of muscle section. This data-processing protocol yielded 16 values for each type of muscle. Capillary-to-fiber ratio (C/F) was also calculated. To evaluate possible differences in fiber dimensions between the two pre-incubation temperatures, cross-sectional area and perimeter of two hundred fibers for each muscle, from four randomly selected fields, were measured on photographs by means of a digitizer table (Calcomp 23180-4; Calcomp, Anaheim, CA) connected to a personal computer using suitable software (Sigma-Scan; Jandel Scientific, Erkrath, Germany). Mean values and standard error were calculated from all the data obtained for each muscle.

Owing to the very dark staining of the Type II fibers in M. extensor metacarpi and M. pronator sections, samples from these muscles were photographed to show the difficulties in counting the capillaries around this fiber type.

All morphological data were obtained from micrographs of different muscle sections taken at a magnification of × 200 by using a light microscope (Dialux; Leitz, Wetzlar, Germany) equipped with a camera (Wild MPS51; Wild, Heerbrugg, Switzerland). A micrograph of a stage micrometer was recorded for calibration.

To check the normality of distribution of sample data, a Kolmogorov–Smirnov test (Lilliefors table) was performed. In case of normality, comparisons between the two temperature fixation sets were carried out by a paired Student's *t*-test, or by a Wilcoxon matched-pair signed rank test in other case. To test the difference in all the parameters measured using the original instead of the modified method, the following ratio was calculated for each pair of serial adjacent sections:

$$T4/T25 = \frac{\textit{Parameter measured at } T=4°C}{\textit{Parameter measured at } T=25°C}$$

Means of this ratio were calculated for M. triceps, M. pectoralis, and M. gastrocnemius. To determine whether the undercounting observed at 4°C is a muscle-dependent value or rather is due only to methodological causes, the following procedure was carried out: T4/T25 ratio data for capillary density were normalized using the transforming $\arcsin(\sqrt{x})$ function (Sokal and Rholf, 1981), which allows a direct application of a one-way ANOVA. Finally, a linear regression for all data from all muscles obtained by both staining techniques was calculated.

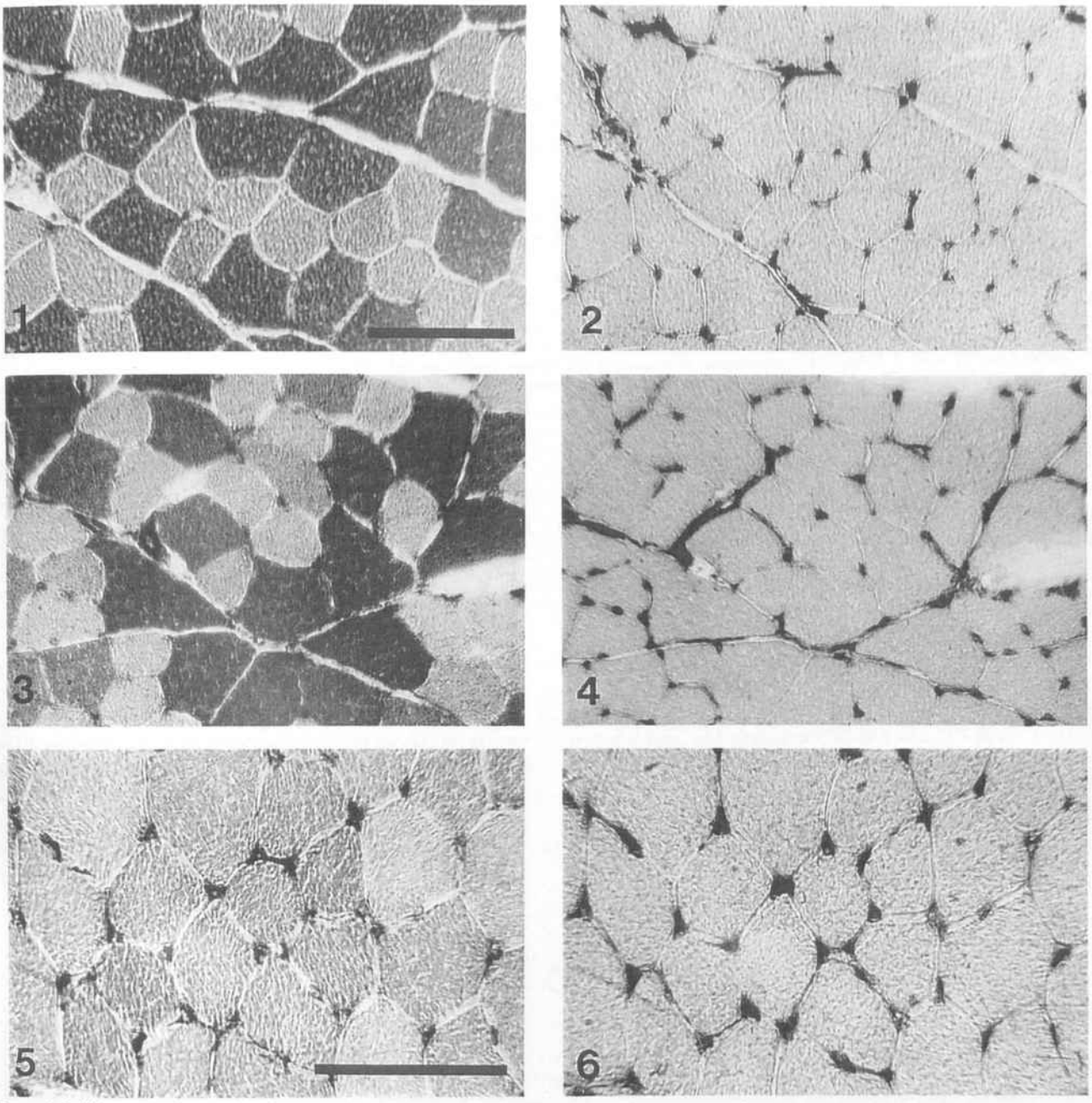
Results

In muscles having Type II fibers in which the histochemical m-ATPase staining was very dark (M. extensor metacarpi and M. pronator), differences between the two pre-incubation temperatures were sharply demonstrated in micrographs, as can be seen in Figures 1–4 where the same field sections of these two muscles are shown after processing by the original and modified methods. These micrographs clearly showed that dark fibers produce a total or partial masking effect over their surrounding capillaries, making it especially difficult to count capillaries between two dark fibers. Because these differences in capillary density estimation between sections pre-incubated at 4°C and 25°C were evident, they were not calculated for these muscles.

In the other three muscles used for this study (M. pectoralis, M. triceps, and M. gastrocnemius) dark fibers were only slightly stained or absent, as can be observed in Figures 7–12, where the same field of serial adjacent sections is shown for each pre-incubation temperature set. This allowed the calculation of the capillary density in the same fields from serial adjacent sections at both pre-incubation temperatures and comparison of the results obtained in each case.

In all of the 48 fields in which capillaries were counted (16 fields for each of the three muscles) the count was always lower in sections pre-incubated at 4°C than in those pre-incubated at 25°C. Table 1 shows the means of the capillary densities for each muscle grouped in the two sets of sections, and it can clearly be deduced that sections pre-incubated at 4°C have lower capillary densities than those pre-incubated at 25°C. If a paired mean values comparison test is performed between data obtained from the two pre-incubation temperature sets, significant differences are observed for each muscle with the very low probability values shown in Table 1. T4/T25 ratio mean values seem to indicate that there is an almost constant capillary undercounting in the three muscles when sections are pre-incubated at 4°C. This was confirmed by the ANOVA results ($F = 1.051$; d.f. = 2,45; $p = 0.358$). No significant differences were detected between the three muscles. This allows the calculation of the linear regression equation plotted in Figure 13.

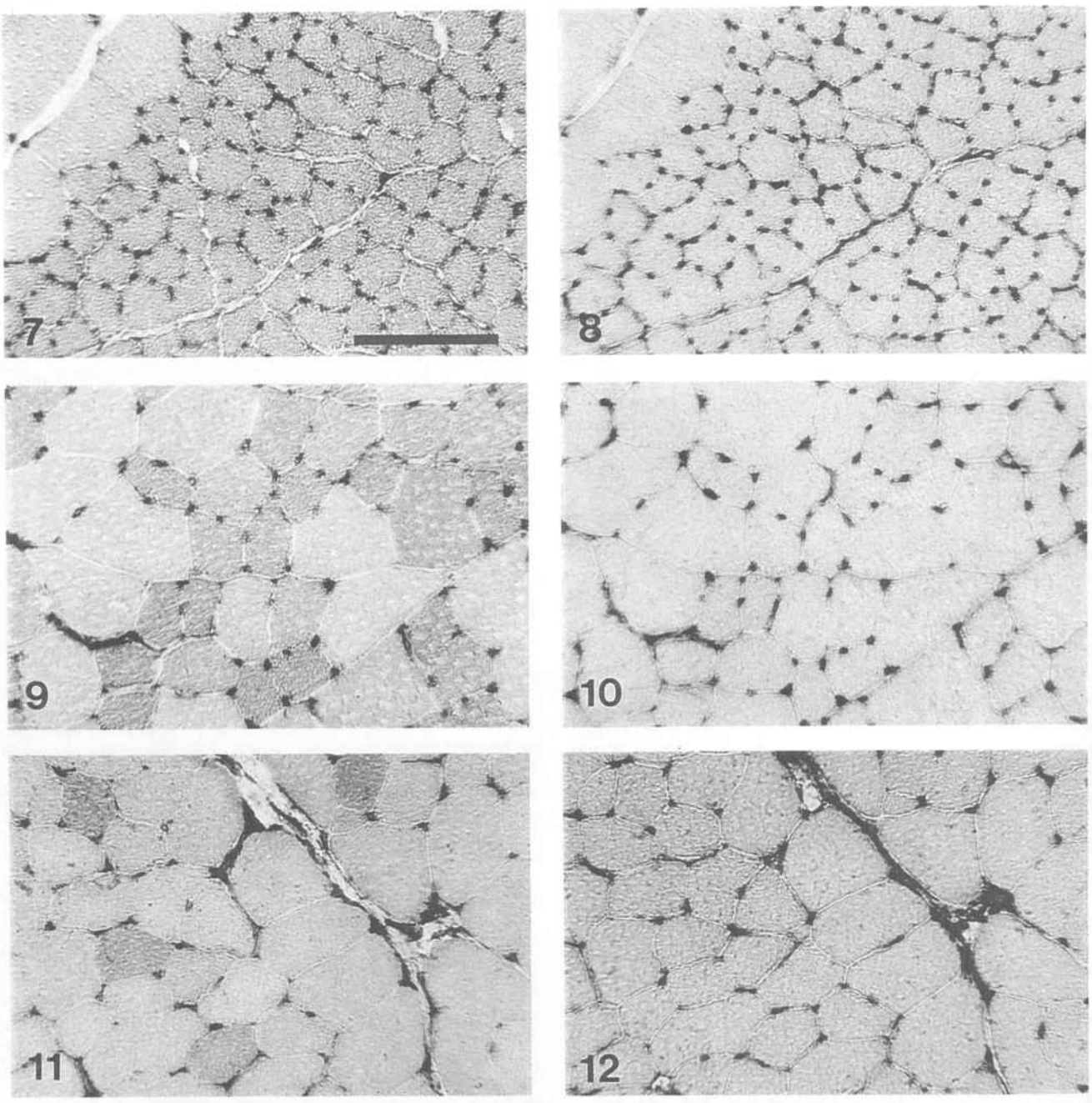
This undercounting on capillary density cannot be explained by changes in fiber size or sectional area, as can be deduced from values in fiber density (Table 1), which are virtually equal after the two pre-incubation temperatures, and also from measured values



Figures 1–6. Micrographs from serial adjacent sections of the same field from pigeon muscles processed after pre-incubation at 4°C (Figures 1, 3, and 5) and 25°C (Figures 2, 4, and 6). Figures 1 and 2 are from M. extensor metacarpi radialis. Figures 3 and 4 are from M. pronator superficialis. Figures 5 and 6 are from M. triceps scapulohumeralis. Original magnifications: Figures 1–4 × 200; Figures 5 and 6 × 500. Bars: Figures 1–4 = 100 μm; Figures 5 and 6 = 50 μm.

of cross-sectional fiber area and perimeter in the different muscles (Table 2). Because of the stability of fiber density values after both procedures, capillary-to-fiber ratio values maintain a similar trend, as do capillary density values (Figure 14 and Table 1).

Capillary density undercounting is not the only difference between the original and modified methods. There is also a remarkable qualitative difference between sections stained after 4°C and 25°C pre-incubation temperatures, as can be seen in Figures 7–12 and in Figures 5 and 6, where the same field sections are shown at high magnification. The capillary cleanness, and even its presence, is better seen in sections pre-incubated at 25°C than in those pre-incubated at 4°C. Thus, in many cases, capillaries that are present in sections processed by the modified method are stained only slightly or not at all in sections pre-incubated at 4°C.



Figures 7–12. Micrographs from serial adjacent sections of the same field from pigeon muscles processed after pre-incubation at 4°C (Figures 7, 9, and 11) and 25°C (Figures 8, 10, and 12). Figures 7 and 8 are from M. pectoralis. Figures 9 and 10 are from M. triceps scapulohumeralis. Figures 11 and 12 are from M. gastrocnemius lateralis. Original magnification × 200. Bar = 100 μm.

Discussion

The present article proposes a modified technique suitable for demonstration of the capillary network in avian skeletal muscle. The method is a modification of that published by Rosenblatt et al. (1987), which is in turn a combination of two previously reported techniques (Meijer and Vloedman, 1980; Meijer, 1970). The histochemical bases of the staining reaction are those reported by Meijer (1970) and Meijer and Vossenberg (1977) and quoted by Rosenblatt et al. (1987). Briefly, ATP present in the incubation medium is hydrolyzed by capillary endothelial ATPase, which is revealed by the deposition of lead sulfide. The formalin fixation medium prevents the sections from wrinkling and shrinkage.

The modification of the method of Rosenblatt et al. (1987) is a change in pre-incubation temperature (25°C instead of 4°C). This apparently slight modification yields marked differences in the final staining, inhibiting the m-ATPase activity of the skeletal muscle fibers, whereas the capillary ATPase activity is not only main-

Table 1. *Comparison of two pre-incubation temperatures on capillary and fiber densities and capillary-to-fiber ratio in different pigeon muscles (mean values ± SE)*[a]

	T = 4°C cap/mm²	T = 25°C cap/mm²	T4/T25 Ratio (%)	t-value Z value	p value
Capillary density (CD)					
M. pectoralis	2115.7 ± 101.8	2361.6 ± 113.0	89.68 ± 0.96	−10.1649	$4.028 \cdot 10^{-8}$
M. triceps	1107.7 ± 60.1	1232.3 ± 73.1	90.20 ± 0.81	−3.5162	$4.378 \cdot 10^{-4}$
M. gastrocnemius	1024.6 ± 56.8	1126.3 ± 65.8	91.36 ± 1.28	−5.7847	$3.600 \cdot 10^{-5}$
Fiber density (FD)					
M. pectoralis	965.1 ± 34.5	969.6 ± 33.5	99.48 ± 0.29	−1.6903	0.0910
M. triceps	579.7 ± 35.3	580.9 ± 34.6	99.70 ± 0.36	−0.5241	0.6002
M. gastrocnemius	601.7 ± 38.6	597.4 ± 38.1	100.70 ± 0.38	1.9789	0.0664
Capillary to fiber ratio (C/F)					
M. pectoralis	2.22 ± 0.12	2.46 ± 0.13	90.16 ± 1.07	−8.9945	$1.977 \cdot 10^{-7}$
M. triceps	1.95 ± 0.09	2.16 ± 0.09	90.49 ± 0.89	−10.3215	$3.290 \cdot 10^{-8}$
M. gastrocnemius	1.72 ± 0.03	1.90 ± 0.05	90.74 ± 1.34	−6.2927	$1.442 \cdot 10^{-5}$

[a] Capillary and fiber density values and capillary-to-fiber ratio for different muscles after pre-incubation temperatures of 4°C and 25°C. Ratio T4/T25 is expressed as percentage. Statistical comparison between mean values was achieved by means of Student's *t*-test for M. pectoralis and M. triceps and by a Wilcoxon rank test for M. gastrocnemius. Significance levels are accepted for $p<0.05$.

tained at this temperature but is also better demonstrated and even enhanced.

The causes of these differences can be only hypothesized, since at present it is not certain which capillary component is responsible for the hydrolysis of ATP. After excluding alkaline phosphatase (Padykula and Herman, 1955), only a few functional properties are known, such as its resistance to acid pre-incubation, sulfhydryl-indifferent behavior in the presence of mercaptide-forming agents (Freiman and Kaplan, 1960), and its activation by several cations (Zn^{2+}, Pb^{2+}, Ca^{2+}, Mg^{2+}). From our results, it can be assumed that a 25°C fixation temperature favors the capillary ATPase staining, which evidences a marked thermosensitivity of the ATPase activity from this endothelial factor. This was observed not only after incubation at 37°C, which gives better results than at 22°C as reported by Rosenblatt et al. (1987), but also after a higher fixation temperature, as our results demonstrate.

The modification of ATPase activity due to changes in pre-incubation temperature has two main consequences: first, avoidance of the masking effect caused by very dark-stained Type II fibers, which allows the counting of the capillaries surrounding these fibers; and second, an increase in the total number of capillaries demonstrated in each section, an effect that is not related to the nature or fiber composition of the muscle studied, the differences between the two methods being consistent and statistically significant in all cases (Table 1). These differences in capillary density values cannot be attributed to thermal shrinkage or swelling of muscle tissue, as can be deduced from values of fiber density, fiber cross-sectional area, and perimeter (Tables 1 and 2). Capillary-to-fiber ratio, a parameter insensitive to alterations in tissue dimensions, further reflects the differences in capillary density obtained by the two procedures (Table 1 and Figure 14).

The bias associated with the original method of Rosenblatt et

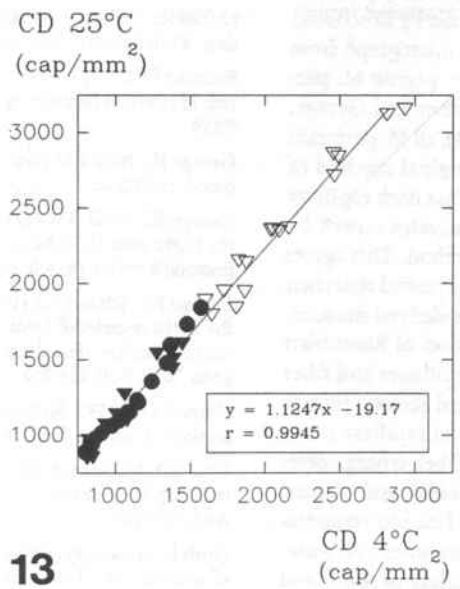


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Figure 13. Relationship between capillary density values obtained by the two methods for M. pectoralis (▽), M. triceps (•), and M. gastrocnemius (▼). Regression equation values are included on the graph.

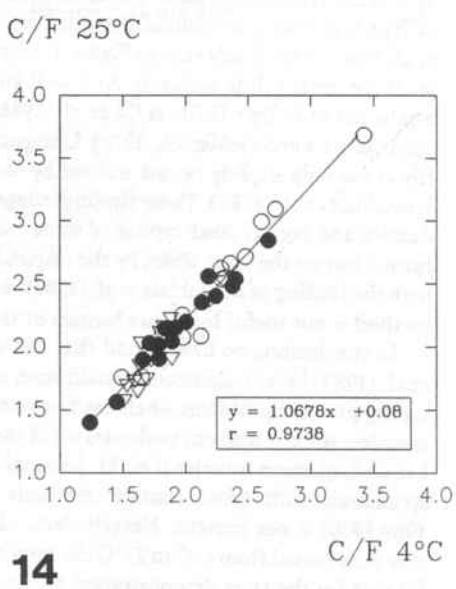

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Figure 14. Relationship between capillary-to-fiber ratio data obtained by the two methods for M. pectoralis (○), M. triceps (•), and M. gastrocnemius (▽). Regression equation values are included on the graph.

Table 2. *Comparison of two pre-incubation temperatures on cross-sectional area and perimeter of fibers from different pigeon muscles (mean values ± SE)*[a]

	T = 4°C cap/mm²	T = 25°C cap/mm²	T4/T25 Ratio (%)	t-value Z value	p value
Fiber area (μm²)					
M. pectoralis	1206.2 ± 70.9	1204.9 ± 68.5	99.94 ± 0.79	−0.5015	0.6160
M. triceps	2089.9 ± 61.7	2082.5 ± 63.2	101.29 ± 0.63	0.6279	0.5308
M. gastrocnemius	1796.2 ± 39.1	1799.3 ± 40.3	100.34 ± 0.61	−0.2915	0.7710
Fiber perimeter (μm)					
M. pectoralis	127.05 ± 2.90	127.03 ± 2.83	100.03 ± 0.42	−0.6076	0.5434
M. triceps	175.48 ± 2.98	174.52 ± 2.99	100.75 ± 0.34	1.7044	0.0898
M. gastrocnemius	161.79 ± 2.00	161.89 ± 2.01	100.07 ± 0.33	−0.1785	0.8586

[a] Cross-sectional fiber area and perimeter values for different muscles after pre-incubation temperatures of 4°C and 25°C. Ratio T4/T25 is expressed as percentage. Statistical comparison between mean values was achieved by means of Student's *t*-test for M. pectoralis and M. triceps and by a Wilcoxon rank test for M. gastrocnemius. Significance levels are accepted for $p<0.05$.

al. (1987) is clearly reflected in the T4/T25 ratio, and values obtained from this comparative index are homogeneous for the entire range of capillary densities in all the muscles studied, as can be deduced from ANOVA results. The absence of significant differences in undercounting capillary densities between the three muscles demonstrates that the method is consistent, since the differences obtained for the two pre-incubation temperatures are not subject to any specificity linked to the characteristics of the muscles, but rather are due to methodological causes.

The high range of capillary density values was due to the fact that fields for sampling were randomly chosen, and differences in capillary densities between fields are associated with the well-known spatial structural heterogeneity of muscle (George and Talesara, 1960; George and Naik, 1959). This wide scope of capillary density values, in addition to the above mentioned homogeneity in the T4/T25 index, permits the calculation of a regression equation that relates the results obtained by the two methods and allows the interpolation of corrected values for data (Figure 13).

An additional difficulty was found when the original method of Rosenblatt et al. (1987) was developed for skeletal bird muscles: Type II fibers present in some bird muscles are not as darkly stained as Type II fibers from mammalian muscles reported by Rosenblatt et al. This can be clearly seen in Figure 7, where a micrograph from an M. pectoralis slide is shown. As is well known, pigeon M. pectoralis has only Type II fibers (Li et al., 1988; Rosser and George, 1986; Talesara and Goldspink, 1978). Unexpectedly, all M. pectoralis fibers are only slightly or not stained by the original method of Rosenblatt et al. (1987). These findings suggest that both capillary density and correct fiber typing of some bird muscles cannot be carried out on the same slides by the original method. This agrees with the finding of Rosenblatt et al. (1987), who reported that their method is not useful for either human or rabbit skeletal muscles.

In conclusion, we have found that the method of Rosenblatt et al. (1987) for simultaneous visualization of capillaries and fiber typing presents problems when used in some bird skeletal muscle samples, since it leads to undercounts of the total capillary number and, in some muscles (i.e., M. pectoralis), fiber-typing correspondence with other classical methods (Brooke and Kaiser, 1969,1970) is not present. Nevertheless, when fixation temperature is increased from 4°C to 25°C the method becomes a very useful tool for the clear demonstration of the capillary network and allows the calculation of several capillary parameters and other morphometrical ones such as fiber area, fiber perimeter and diffusion distances (Viscor et al., 1992).

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II

A myosin ATPase and acetylcholinesterase combined histochemical method for the demonstration of fiber types and their innervation pattern in skeletal muscle.
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Histochemistry

A combined myosin ATPase and acetylcholinesterase histochemical method for the demonstration of fibre types and their innervation pattern in skeletal muscle

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Abstract. An improved, combined staining method for myofibrillar ATPase (m-ATPase) and for acetylcholinesterase activity is described. This method allows the observations, on the same slide, of the classical histochemical m-ATPase profile following the Brooke and Kaiser technique and the neuromuscular junction morphology. Thus the pattern of innervation, nerve ending structure and number of nerve endings along the fibres is shown simultaneously for the basic differentiation between slow and fast fibres. The use of acidic and alkaline preincubation allows better visualization of endplate morphology and avoids the masking effect of a positive m-ATPase reaction. The technique has been validated on skeletal muscles from avian and mammalian species.

Introduction

In order to show the structure and the number of nerve endings, many histochemical techniques have been developed. Most of them (Naik 1963; Karnovsky and Roots 1964; Gwyn and Heardman 1965; Henderson 1967; Namba et al. 1967; Toop 1976) are modifications of the original Koelle and Friedenwald (1949) cholinesterase histochemical method. The various methods all reveal the acetylcholinesterase (AChE) activity sites of the subneural apparatus on the muscle fibre sarcolemma by means of the deposition of a brownish silver sulphide precipitate.

Despite the great number of studies using these techniques to demonstrate the nerve ending structure, few combine AChE detection with other histochemical methods. To our knowledge, the only published reports are the paper of Ogata (1965), which combines succinic dehydrogenase and AChE staining, and that of Ashmore et al. (1978), using a myosin ATPase (m-ATPase) technique with an AChE method. In the present study, we have developed a combined m-ATPase and AChE method to show, on the same slide, both the kind of nerve ending and the fibre type, after either acidic or alkaline preincubation. The latter permits the use of the Brooke and Kaiser (1970) classification of fibre type for mammals and the analogous Barnard et al. (1982) one for birds.

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Materials and methods

Four Sprague-Dawley rats, four urban pigeons (*Columba livia* var. *domestica*) and four mallard ducks (*Anas platyrhynchos*) were used for this study. After anaesthetizing the animals with sodium pentobarbital (75 mg/kg body weight) the following muscles were dissected out: m. extensor digitorum longus (EDL) and m. soleus of the rats (Greene 1959); and m. iliotibialis cranialis (ITC), m. pectoralis and m. anterior latissimus dorsi (ALD) of both avian species (Vanden Berge 1979). All samples were immediately frozen in isopentane cooled to −160° C and then stored in liquid nitrogen until sectioning. Longitudinal and transverse sections (25 μm thick) were cut for each muscle in a cryostat (Frigocut, Reichert-Jung, Heidelberg, Germany) at −22° C and mounted on gelatinized slides. Sections were separately processed in one of two ways: (I) a modification of the Brooke and Kaiser m-ATPase technique (steps 1 to 4 of the protocol explained below) and (II) the combined m-ATPase and AChE method proposed here (steps 1 to 5).

The complete protocol comprised the following steps. (1) Fixation (5 min) in Guth and Samaha (1970) buffered fixative at 4° C (144 m*M* sodium cacodylate, 336 m*M* sucrose, 68 m*M* $CaCl_2$ and 5% formalin). (2) Rinsing 15 times in distilled water. (3) The Brooke and Kaiser (1970) technique for m-ATPase was then applied as modified by Reichmann and Pette (1982), but preincubation was performed for only 5 min in the acidic media (pH 4.0, pH 4.2, pH 4.3) and for 10 min for alkaline ones (pH 10.6, pH 11.0). (4) Rinsing for 15 min in running tap water. (5) Toop's (1976) AChE method was then developed with some modifications as follows:

(a) Incubation in AChE medium (Page 1971) at pH 5.5 for 30–45 min and 39° C for bird muscles and 10 min at 37° C for rat muscles. The concentration of the AChE medium was also varied depending on the muscles processed: when rat muscles were incubated, a medium containing 0.5 mg/ml of acetylthiocholine iodide was used, whereas for bird muscles 1 mg/ml gave better results.

(b) Rinsing five times with distilled water.

(c) Incubation in 0.5% $K_3Fe(CN)_6$ for 10 min at room temperature.

(d) Fixation of sections for 30 min at room temperature in buffered formol-calcium with cadmium and magnesium (Namba et al. 1967), and adjusted to pH 7.0 with 2 *N* NaOH.

(e) Washing with repeated changes of distilled water for 20 min.

(f) Incubation in 5% aqueous $AgNO_3$ containing 0.1% $CuSO_4 \cdot 5H_2O$ for 30 min at 39° C for bird muscles or 37° C for rat ones; a small amount of $CaCO_3$ should be placed on the bottom of the staining jar.

(g) Rinsing five times in distilled water.

(h) Development in a solution of 1% hydroquinone and 5% Na_2SO_3 in distilled water for 10 s.

(i) Rinsing three times in distilled water.

(j) Fixation in 5% sodium thiosulphate for 2 min.

(k) Rinsing three times in distilled water.

(l) Mounting in a glycerine droplet.

Micrographs of different muscle sections were taken at magnifications ×80 and ×200 using a light microscope (Dialux, Leitz, Wetzlar, Germany) equipped with a camera (MPS51, Wild, Heerbrugg, Switzerland). A micrograph of a stage micrometer was recorded on each film for calibration. Serial sections processed by both protocols were compared on micrographs taken from the same fields of view in order to ensure the absence of differences in the Brooke and Kaiser histochemical profile after the application of either procedure I or II.

Results

Demonstration of the staining for AChE activity, in combination with the application of the m-ATPase technique, did not alter the m-ATPase histochemical profile, since no differences in fibre typing were found between staining protocols I and II. The muscle fibres were suitably stained depending on their acid and alkaline stabilities. The subneural apparatus was stained as a brownish-black silver sulphide precipitate, which denotes zones of high AChE activity. Some differences in the intensity of the AChE staining were found between rat and bird nerve terminals and also between type II and type I or III nerve endings of bird muscles. Thus some modifications to the incubation times and to the final acetylthiocholine iodide concentration in Page (1971) medium became necessary. In soleus and EDL rat muscles the staining intensity of the subneural apparatus was very high. For this reason better results were obtained using an incubating medium with an acetylthiocholine iodide concentration of 0.5 mg/ml, which was one-half of that used for bird muscles. The time of incubation was also important; thus, in rat muscles, incubation for 10 min was sufficient to develop a suitable deposition of silver sulphide, whereas in avian species 30 min were required for muscles containing type II fibres and 45 min when type I or III fibres were present.

Four fibre types were identified according to their m-ATPase profile: type I (acid stable/alkali labile) were found in both rat muscles and in ITC of the two birds; type II (acid labile/alkali stable), which can be subdivided into types IIA and IIB, were present in the rat muscles studied and also in bird pectoralis and ITC; type III (acid stable/alkali stable) were exclusively found in ALD muscles of both avian species.

The innervation pattern shows great differences depending on the fibre type and vertebrate group considered. The most apparent difference was found between type I fibres. In bird muscles these fibres present multiple innervation with nerve structures ending as a series of small light knobs (Fig. 1), which differ considerably from the black, intensely stained end plates with horseshoe-like structures or buttons of rat type I fibres (Fig. 2). In transverse sections these buttons are seen as highly localized AChE activity sites on the fibre perimeter (Fig. 3), a result quite different from the transverse view of the bird type I fibres (Fig. 4). Moreover, multiple innervated rat type I fibres were not detected.

Rat type II fibres present the same nerve ending structure and innervation pattern as rat type I. Type II fibres of bird muscles also present focal or single innervation but their nerve terminal structure was quite different from that of the rat. The end plate is represented as longitudinal finger-like extensions (Fig. 5) in contrast to the localized buttons observed in the rat (Figs. 2 and 6). In addition, in transverse sections the end plate region extends around a large part of the fibre perimeter (Fig. 4) instead of occupying a localized zone, as is the case of mammalian end plates (Fig. 3). Type III fibres detected only in avian ALD muscles have their nerve endings with "en grappe" structures and are multiply innervated (Figs. 7 and 8).

Discussion

The technique proposed in the present study offers some improvements over the previous method for visualization of combined m-ATPase and AChE reported by Ashmore et al. (1978). The most important difference between the two methods is the m-ATPase reaction developed in each case. Our method follows the classical Brooke and Kaiser (1970) method of m-ATPase staining as modified by Reichmann and Pette (1982), which involves both acidic and alkaline preincubations. By comparison, Ashmore et al. (1978) use only acidic preincubation (formic acid fixation), and this difference has important implications.

When acid stable/alkali labile fibres are darkly stained at acidic pH, the patterns of innervation are difficult to identify due to the masking effect produced by the staining of m-ATPase over the staining of AChE; moreover, the latter is lighter in these slow fibres. To resolve the lack of distinction between m-ATPase and AChE, alkaline preincubated slides are very useful, due to the absence of m-ATPase staining in slow type I fibres at high pH and thus clearly revealing AChE staining at the nerve endings. Fast fibres, which show a dark staining reaction when preincubated in alkaline conditions, are not masked in this way due to their greater AChE activity, clearly showing the nerve ending structure.

The above-mentioned differences in AChE activities between fast-twitch and tonic muscles have been noted previously (Buckley and Heaton 1971; Lyles and Barnard 1980). Lyles and Barnard (1980) suggested that these differences are due to the absence of a heavy form of AChE in tonic muscles, which gives them a much lower sensitivity to acetylcholine. The reduction of the $AgNO_3$ concentration (5% instead of 20%), to avoid the interference of silver precipitates as small black

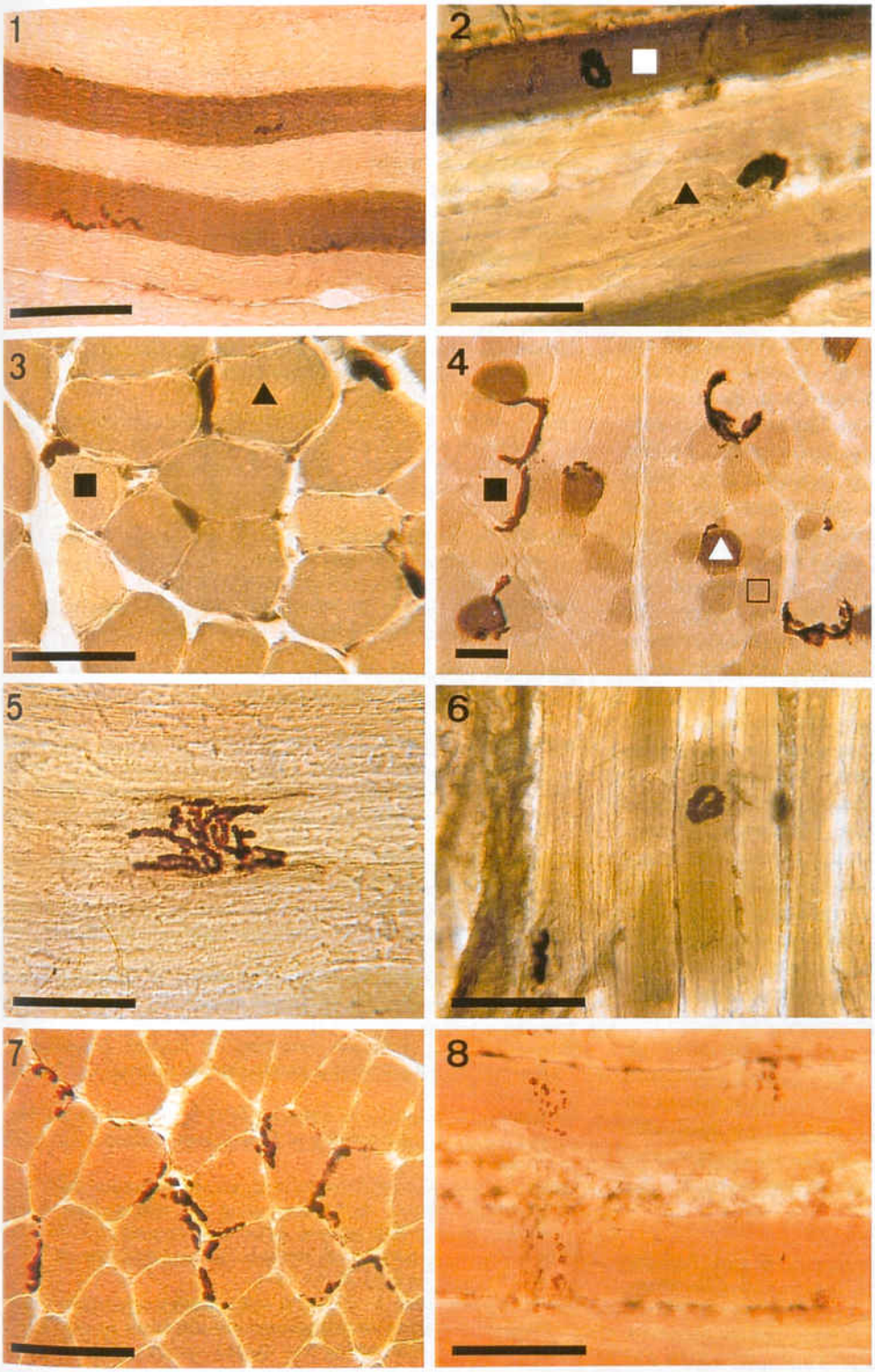

Fig. 1. Longitudinal section of pigeon iliotibialis cranialis at pH 4.2. Note two multiple innervated type I fibres. **Fig. 2.** Longitudinal section of rat soleus at pH 11.0. The *square* indicates type II fibre and *triangle* indicates type I. **Fig. 3.** Transverse section of rat soleus at pH 4.2. The *square* indicates type II fibre and *triangle* indicates type I. **Fig. 4.** Transverse section of pigeon iliotibialis cranialis at pH 4.2. The *filled square* indicates type IIA fibres, *open square* indicates type IIB and *triangle* indicates type I. **Fig. 5.** Longitudinal section of pigeon pectoralis at pH 4.2. Detail of finger-like end plate structure of a type II fibre. **Fig. 6.** Longitudinal section of rat extensor digitorum longus at pH 4.2. Frontal and lateral view of a type II nerve ending. **Fig. 7.** Transverse section of type III fibres of pigeon anterior latissimus dorsi at pH 11.0 with their innervation pattern. **Fig. 8.** Longitudinal section showing multiple innervation of type III fibres of mallard anterior latissimus dorsi. All *bars* represent 100 μm

grains with the brownish-black depositions of the nerve endings, has also improved the Ashmore et al. (1978) combined technique. The nomenclature followed in this study was proposed by Barnard et al. (1982) for bird muscles, which is a modification of that proposed by Brooke and Kaiser (1970) for mammalian species. This nomenclature is more widely accepted than that followed by Ashmore et al. (1978) and should thus be used preferentially for classification of vertebrate muscle fibres.

This combined method may be useful for researchers who develop histochemical assays in many different fields. Thus it could be used in studies involving comparative physiology, in order to establish the skeletal muscle fibre type characteristics of different vertebrate groups. As our results show, there are differences in the innervation patterns of fibres with identical histochemical profiles (type I and II) between rat and bird muscles. Differences in type I fibres were already reported some years ago (Barnard et al. 1982) but the controversy about the physiological character of non-mammalian muscle type I fibres is still unresolved (Rosser and George 1986; Hikida 1987; Mutungi and Johnston 1987; Hayashi and Nagata 1991; Wilkinson et al. 1991; Meyers 1992). The present method could help to resolve this and other questions, if it is properly combined with physiological techniques.

The method described here may also be used in studies determining the change in histochemical properties of cross-innervated, re-innervated, self-innervated or denervated muscles and in those dealing with alterations in end plate architecture after aging. The feature of combined m-ATPase and AChE staining gives it a special utility in studies on neuromuscular formation and fibre differentiation during avian embryogenesis, mammalian fetal and neonatal development, and the histochemistry of neuromuscular formation. Pathologists will find this combined m-ATPase and AChE method useful in a variety of different scenarios: for studying alterations in disease states involving motor nerve terminal degeneration, in complementary studies concerning myosin expression in dystrophic muscles, for routine staining methods in the case of congenital myasthenia, and in other musculoskeletal diseases where both m-ATPase histochemistry and nerve terminal visualization could be necessary for diagnostic purposes.

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III

Innervation distribution pattern, nerve ending structure and fiber types in pigeon skeletal muscle.
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Innervation Distribution Pattern, Nerve Ending Structure, and Fiber Types in Pigeon Skeletal Muscle

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ABSTRACT **Four fiber types have been characterized in different pigeon skeletal muscles according to their innervation pattern (nerve ending structure and innervation distribution) and histochemical properties (SDH and m-ATPase activities). All fast fibers, types IIA and IIB, present aggregated distribution of their nerve endings with "en plaque" structures and very low innervation frequencies. The two kinds of slow fibers recognized are multiple innervated and present higher innervation frequencies. However, type I fibers have nerve terminals in small knobs with uniform localization, whereas type III fibers present "en grappe" nerve endings, which tend to be randomly distributed. Fiber type composition of skeletal muscles has been found closely related to their biomechanical function. Fast fibers are predominant in muscles with an active role in locomotive movements, whereas slow fibers are mainly or exclusively located in postural muscles.** © 1993 Wiley-Liss, Inc.

Key words: Skeletal muscle, m-ATPase, Acetylcholinesterase, Histochemical method, Innervation pattern, Fiber types, Bird, Mammal

During the last three decades many histochemical methods have been developed to classify the different skeletal muscle fiber types on the basis of their enzymatic activities. Methods most commonly used are those for myosin ATPase (Padykula and Herman, 1955; Brooke and Kaiser, 1970; Guth and Samaha, 1970) and oxidative (Nachlas et al., 1957) and glycolytic (Wattenberg and Leong, 1960) enzymes.

After combining histochemical with biochemical and physiological methods, skeletal muscle fiber classifications have been established for mammals (Peter et al., 1972; Brooke and Kaiser, 1970; Guth and Samaha, 1970; Burke et al., 1973). The more widespread of them accepted the existence of fast- and slow-twitch fibers. The presence of slow-tonic fibers must be considered when classifying skeletal muscle fibers of non-mammalian vertebrates (Nene, 1977; Gleeson et al., 1980; Barnard et al., 1982; Rosser and George, 1984, 1985; Mutungi, 1990). Tonic fibers do not appear in mammalian locomotory muscles, although they have been found in their oculomotor (Zenker and Anzenbacher, 1964; Kaczmarski, 1974; Pierobon Bormioli et al., 1980), vocal (Rossi, 1990), and middle ear muscles (Mascarello et al., 1983).

There are many differences between tonic and twitch fibers: tonic fibers do not respond with a propagated action potential to a single nerve stimulation, require several seconds to reach maximum tension, and develop sustained contraction after application of depolarizing agents such as acetylcholine and K^+; conversely, twitch fibers give acetylcholine and K^+ contractions of short duration, propagate an action potential, and exhibit a rapid rise in tension after nerve stimulation (for review see Morgan and Proske, 1984). In addition to these physiological differences, there are many structural and histochemical ones (Ashmore et al., 1978; Rouaud and Toutant, 1982; Morgan and Proske, 1984; Johnston, 1985). Among these, the most remarkable and the earliest studied are the nerve ending structure and the number of synaptic contacts along a single fiber. On the basis of these features, tonic fibers are multiply innervated and have "en grappe" nerve endings whereas twitch fibers are focally innervated and have "en plaque" end-plates (Morgan and Proske, 1984, and references therein).

In addition to tonic fibers, a histochemically mammalian-like slow type has been described in bird muscles (Barnard et al., 1982; Hikida, 1987). These fibers have been less documented than tonic ones and their possible twitch character is unresolved.

Considering all this, the aim of this paper is to contribute toward establishing differences in the histochemical profile and, especially, the nerve ending structure and distribution between different fiber types of pigeon skeletal muscle.

MATERIALS AND METHODS

Six urban pigeons caught in Barcelona were used for this study. Before the removal of muscles, pigeons were

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anesthetized with sodium pentobarbital (75 mg/kg body weight) and the following muscles were dissected out: M. iliotibialis cranialis (ITC), M. scapulohumeralis (SCH), M. pectoralis (PECT), M. brachialis (BRC), M. anterior latissimus dorsi (ALD), and M. serratus metapatagialis (SMP), according to Vanden Berge (1979) nomenclature. All samples were immediately frozen in isopentane cooled to −160°C and then stored in liquid nitrogen until sectioned. Longitudinal and transverse sections (25 μm thick) were cut for each muscle in a cryostat (Frigocut, Reichert-Jung) at −22°C and mounted in gelatinized slides.

Sections were processed in two ways: 1) sections were first fixed in Guth and Samaha (1970) buffered fixative at 4°C (sodium cacodylate 0.144 M, sucrose 0.336 M, $CaCl_2$ 0.068 M, and 5% formalin) and then the histochemical technique for m-ATPase of Brooke and Kaiser (1970) as modified by Reichmann and Pette (1982) was developed, but performing preincubation only for 5 minutes for acidic media (pH 4.0, pH 4.2, pH 4.3) and for 10 minutes for alkaline ones (pH 10.6, pH 11.0); 2) a combined m-ATPase and AChE method (Torrella et al., 1993) was developed. Briefly, the Brooke and Kaiser (1970) m-ATPase technique as described in the first procedure was developed. Then, after rinsing 15 minutes in running tap water, the Toop's (1976) histochemical AChE procedure was developed with some modifications as to the time of incubation in AChE medium (30–45 minutes instead of 9–12 minutes) and the concentration of aqueous $AgNO_3$ medium (5% instead of 20%)

Serial adjacent sections were also processed for the demonstration of succinic dehydrogenase enzyme activity to determine the oxidative character of the fibers, with some modifications over the original technique (Nachlas et al., 1957) previously described (Viscor et al., 1992).

Micrographs of different muscle sections were taken at magnifications ×80 and ×200 by using a light microscope (Dialux, Leitz) equipped with a camera (MPS51, Wild). A micrograph of a stage micrometer was recorded in each film for calibration. Serial sections processed by both protocols were compared on micrographs from the same field in order to ensure the absence of differences in the Brooke and Kaiser histochemical profile after the application of either the first or second procedures.

Qualitative differences of innervation patterns, nerve ending structure, and histochemical profiles were tested on micrographs taken from contiguous fields in transverse and longitudinal sections. To quantify frequencies of innervation only micrographs of transverse sections were used. In large muscles (PECT, ITC, and anterior slip from SMP), fields were taken in order to form a transection band through the whole muscle from superficial to deep zones in PECT, and from anterior to posterior zones in ITC and anterior SMP. In small muscles (ALD, BRC, and posterior slip from SMP) all muscle fibers were included.

Fields were chosen in order to always delimit similar areas, with a number of fibers ranging from 400 to 550, according to the different fiber type proportions present in each muscle and their different areas. Innervation frequencies were calculated as percentage of fibers presenting AChE activity of the total number of fibers considered. This percentage was calculated in two ways: first, we calculated innervation frequency for each field and fiber type ($IF_{(i)t}$), and then we calculated the global innervation frequency for each muscle sample and fiber type (IF_t), which was performed taking in consideration data from all fields previously chosen. For this purpose, the following formula was calculated for each muscle sample:

$$IF_t = \frac{\sum_{i=1}^{N} (n_{(i)t} \cdot IF_{(i)t})}{\sum_{i=1}^{N} n_{(i)t}}$$

where N = number of fields and $n_{(i)t}$ = number of fibers of type t into field i.

Values obtained for $IF_{(i)t}$ and IF_t were separately analyzed in order to test two different aspects.

First, we tested whether there were significant differences in innervation frequency between the muscles studied for each fiber type. Furthermore, global differences between fiber types were also contrasted. All this was done by applying a normalizing transformation (Sokal and Rohlf, 1981) over IF_t parameter:

$$T(IF_t) = \arcsin \sqrt{\frac{IF_t}{100}}$$

where $T(IF_t)$ is the transformed value.

Two one-way ANOVA and multiple range tests (Scheffe's procedure) were tested over $T(IF_t)$ values.

Second, $IF_{(i)t}$ values were used in order to show fiber innervation distributions for each fiber type, which was done by comparing average and variance values.

RESULTS

The characteristics of the four different skeletal muscle fiber types found in the muscles used for this study are given in Table 1. We found two slow fiber types (I and III) and two fast ones (IIA and IIB), the distribution of which among muscles studied is summarized in Table 2 and described as follows.

M. anterior latissimus dorsi (ALD) and M. brachialis (BRC)

Both muscles are homogeneous according to their fiber types. After the development of m-ATPase reaction, all fibers present in these muscles are type III, having acid stable/alkali stable reactions (Figs. 1A,B) and moderate SDH activities (Fig. 1C). In longitudinal sections, multiply innervated fibers and "en grappe" nerve terminals can be seen (Fig. 2A) and, under low magnification, a high innervation density through the whole of both muscles is clearly visible. In transverse sections, this can also be demonstrated due to the higher probability of finding innervated fibers along the whole muscle transverse area, after sectioning at different levels in the muscle (Fig. 2B). This high innervation density is shown in Table 3, where also it can be seen that BRC and ALD have similar frequencies of innervation.

M. pectoralis (PECT)

From transverse sections, only fast fibers are present in this powerful muscle, as shown by the intense m-ATPase stain after alkali preincubation (Fig. 1D). These type II fibers can be subdivided into IIA and

TABLE 1. Histochemical profiles and innervation characteristics of skeletal muscle fiber types

	Fiber type			
	I	IIA	IIB	III
ATPase				
alkali preincubation	light	dark	dark	dark
acid preincubation	dark	light	moderate	dark
SDH activity	low	low to moderate	moderate to high	moderate
Innervation pattern	multiple	focal	focal	multiple
Innervation distribution	random	aggregated	aggregated	uniform
End-plate structure	small knobs	"en plaque"	"en plaque"	"en grappe"

TABLE 2. Skeletal muscle fiber type frequencies in different fields of pigeon muscles

	Fiber type			
Muscle	I	IIA	IIB	III
ALD	Absent	Absent	Absent	100%
BRC	Absent	Absent	Absent	100%
ITC	0–15%	51–99%	16–50%	Absent
PECT	Absent	0–15%	85–99%	Absent
$SMP_{(a)}$	Absent	16–50%	51–84%	Absent
$SMP_{(p)}$	100%	Absent	Absent	Absent

$SMP_{(a)}$ = Serratus metapatagialis, anterior slip.
$SMP_{(p)}$ = Serratus metapatagialis, posterior slip.

IIB after acidic preincubation (Fig. 1E) and, in some cases, these two subtypes can also be shown in alkali-preincubated sections as differential staining intensities. Type IIA fibers have greater diameters and areas than IIB and a heterogeneous distribution along the muscle, occupying preferentially the peripheral parts of the bundles and drastically diminishing their number in deep muscle zones. In the pigeon PECT, these IIA fibers are anaerobic whereas IIB fibers are oxidative, as is deduced from SDH assay (Fig. 1F).

On longitudinal sections (Fig. 2C), focally innervated fibers are seen with fingerlike end-plate structures, whereas on transverse sections, no differences can be observed in innervation patterns (Figs. 1E, 2D) or in innervation frequencies (Table 3) between IIA and IIB fibers. Both types show the AChE stain surrounding a great part of the fiber perimeter when the end-plate zone is sectioned and yield lower innervation frequencies than those observed for type I and III fibers of other muscles. Due to the focal innervation pattern of this muscle, there are many fields in which the end-plate presence is scarce or totally absent and others where they are present in great number in specific localized zones.

M. serratus metapatagialis (SMP)

This muscle presents two clearly different slips. The posterior slip is composed purely by type I fibers (acid stable/alkali labile) with low SDH activities. These fibers are multiply innervated with nerve ending structures presenting small dots, as can be observed after longitudinal sectioning (Fig. 2E). On transverse sections, the small knobs or punctuation structures on the edge of the fiber are also evident and these are characterized by a low level of AChE activity. Furthermore, homogeneous innervation frequencies can be seen (Fig. 2F), resembling those observed for tonic muscles, but with slightly lower values (Table 3).

The anterior slip has only type II fibers, which can be classified as IIA and IIB subtypes after acidic preincubation. Correlations between m-ATPase and SDH activities in the fast part of this muscle are similar to those observed in PECT as well as the distribution of IIA fibers in muscle bundles. Nerve endings have "en plaque" structures in both fiber types and low innervation frequencies (Table 3) when the whole muscle is considered, in the same way that in PECT, a focal innervation pattern is evident (Table 3).

M. iliotibialis cranialis (ITC) and M. scapulohumeralis (SCH)

ITC is a mixed muscle presenting three fiber types (Figs. 1G,H,I), the distribution of which is not homogeneous along the muscle: the fiber frequencies change from anterior to posterior parts and from deep to superficial muscle zones. Characteristics of types I, IIA, and IIB fibers of this muscle correlate well with those observed for SMP and PECT. In addition, innervation frequencies when the whole muscle is considered also match well with those obtained for SMP and PECT, with no differences between II subtypes but with strong differences between types I and II (Table 3). Slight differences can be found in SDH activities, being more moderate in type II fibers.

SCH is also a mixed muscle with hetereogeneous distribution of fiber types. Type II fibers are distributed through the whole muscle in different frequencies, while type I fibers are localized in a very specific deep zone of the muscle, being scarce in number. For this reason, SCH has only been taken into consideration to determine the nerve ending structure and its number along fibers, and we have not quantified frequencies of innervation in transverse sections. The nerve ending structure and multiple innervation pattern observed in type I are similar to those present in ITC and posterior slip of SMP, as is its histochemical profile. Properties of type II fibers of SCH also correlate well with the equivalents of ITC, PECT, and SMP.

Statistics

After testing the possible existence of differences in innervation frequencies for each fiber type (IF_t) between muscles studied, by means of a one-way ANOVA test for each fiber type (I, IIA, IIB, and III) and considering one level for each muscle, we have obtained the values shown in Table 4. No significant differences can be seen in any case but a relatively high probability

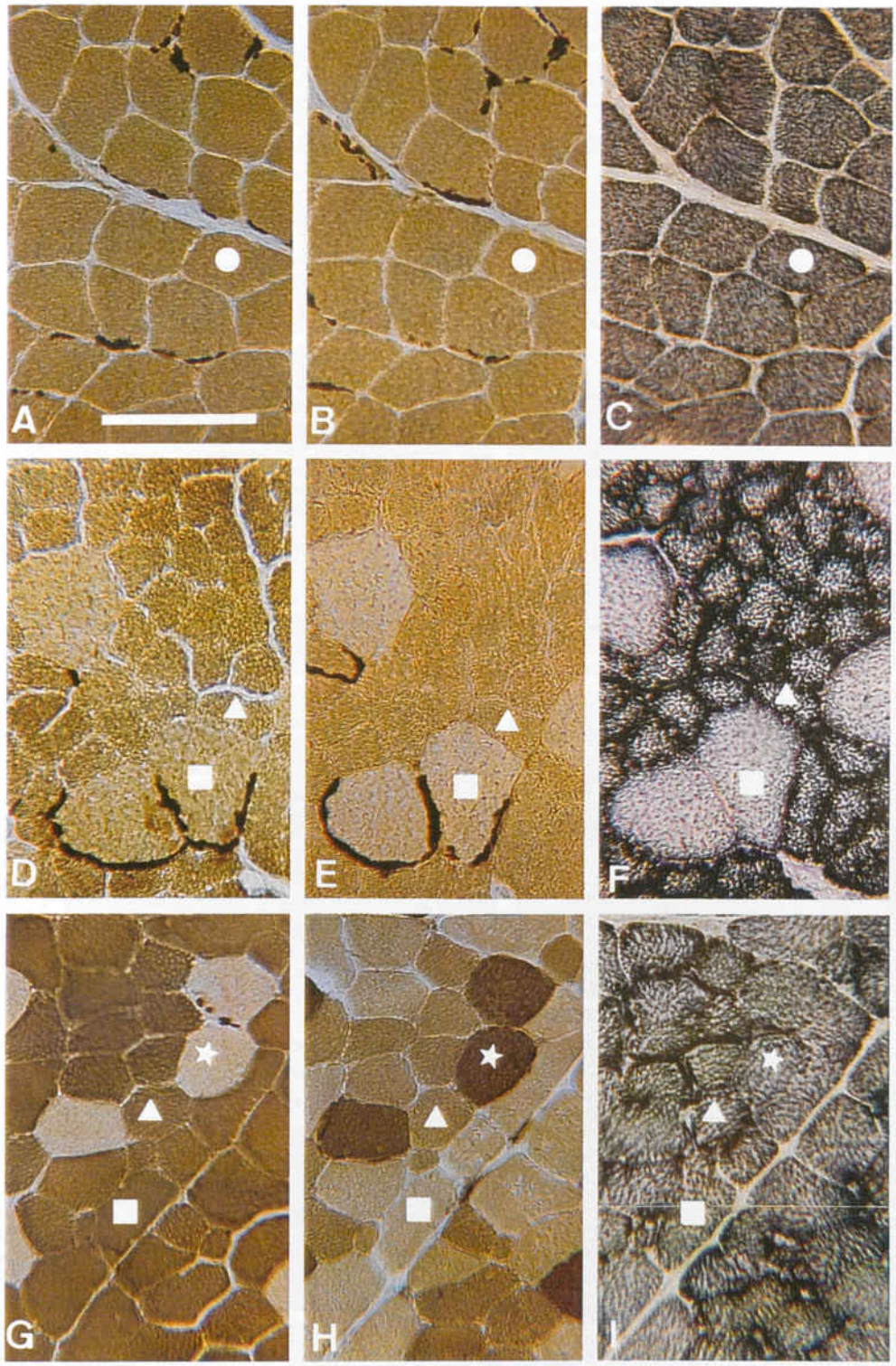

Fig. 1. Transverse sections of different muscles (**A, B, C:** ALD; **D, E, F:** PECT; **G, H, I:** ITC) processed for the combined m-ATPase and AChE method (A, D, and E, preincubation at pH 11.0; B, E, and H, preincubation at pH 4.2), and SDH assay (C, F, and I). Different fiber types are identified on the micrograph. Fiber type codes: I, star; IIA, square; IIB, triangle; III, circle. Note AChE activities surrounding some fibers and differences between nerve ending structures in fibers I, II, and III. Note also the absence of differences between nerve endings of IIA and IIB fibers. Bar represents 100 μm.

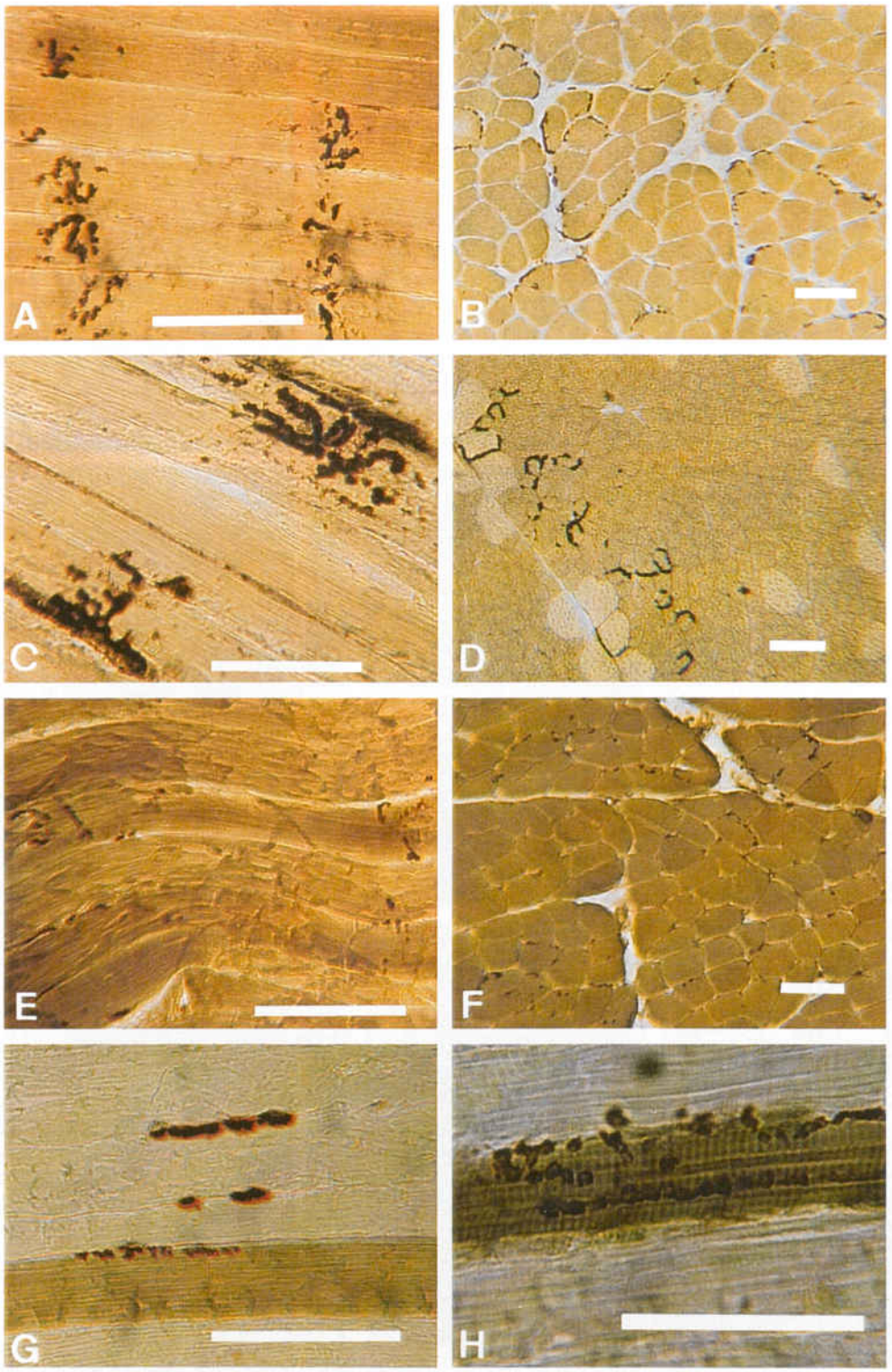

Fig. 2. **A–F:** Longitudinal (A, C, and E) and transverse (B, D, and F) sections of BRC (A and B), PECT (C and D), and SMP (E and F) stained for the combined method after preincubation at pH 4.2. Note nerve endings structure: "en grappe" with multiple innervation (A), "en plaque" with focal innervation (C), and small knobs with multiple innervation (E). Note also the innervation distribution at low magnifications: uniform (B), aggregated (D), and random (F). **G, H:** Longitudinal sections of ITC stained after preincubation at pH 4.2. G: Lateral views of the nerve ending of an innervated type I (dark stain) and a type II (slight stain) fiber. Note the different AChE stain intensity between both fiber types. H: Type I fiber where its nerve ending structure can be seen in detail. Note the series of knobs distributed along the fiber. Thick bar in figure H represents 50 μm, whereas narrow ones in all other figures represent 100 μm.

TABLE 3. Innervation frequency for the different skeletal muscle fiber types in some pigeon muscles

Muscle	Fiber type	n	$\bar{x}$	S^2_{n-1}	Range
ALD	III	4	14.27	2.32	12.50–16.12
BRC	III	5	14.86	2.37	12.91–16.71
PECT	IIA	5	2.28	2.64	1.28–5.16
PECT	IIB	5	1.76	0.37	1.18–2.75
$SMP_{(a)}$	IIA	4	2.64	2.07	1.24–4.30
$SMP_{(a)}$	IIB	4	1.95	0.05	1.71–2.18
$SMP_{(p)}$	I	4	12.73	15.94	9.09–18.10
ITC	I	4	10.04	0.11	9.65–10.47
ITC	IIA	4	2.58	0.53	1.95–3.55
ITC	IIB	4	2.92	0.87	1.61–3.82

TABLE 4. Statistical comparison of the innervation frequencies found in the each fiber type among different muscles

Fiber type (compared muscles)	F	df	P
I ($SMP_{(p)}$ vs. ITC)	1.7960	1, 6	0.2287
IIA (PECT vs. $SMP_{(a)}$ vs. ITC)	0.1689	2, 10	0.8469
IIB (PECT vs. $SMP_{(a)}$ vs. ITC)	3.5351	2, 10	0.0690
III (ALD vs. BRC)	0.3312	1, 7	0.5830

value ($P = 0.069$) is present for IIB fibers. After applying a multiple range test (Scheffe's procedure), it can be seen that this is due to particularities in ITC.

When homogeneity among muscles for each fiber type in IF_t was seen, we considered possible global differences between different fiber types in innervation frequencies. Once more, we applied one-way ANOVA but this time taking one level for each fiber type, and highly significant differences were found ($F_{3,39} = 151.5$, $P < 0.0001$). These results led us to develop a second multiple range test (Scheffe's procedure), the results of which are shown in Table 5. Large differences can be observed between types IIA, IIB and types I, III ($P < 0.01$) and, to a slightly lesser extent, between types I and III ($0.01 < P < 0.05$). However, no significant differences between types IIA and IIB ($P > 0.1$) were found.

Finally, Table 6 shows the distribution pattern of innervation for each fiber type by means of a comparison between average ($\bar{x}$) and variance (S^2) values associated with $IF_{(i)t}$ parameter distributions.

DISCUSSION

The Brooke and Kaiser (1970) nomenclature is extensively used to classify mammalian skeletal muscle fibers. By means of the detection of m-ATPase activity after acidic and alkaline preincubations, fibers are basically classified as type I, IIA, or IIB. Classification problems appear when non-mammalian vertebrates muscles are considered, because of the existence of two types of slow fibers: a veritable slow-tonic type and a not-well characterized slow type with a mammalian-like profile. For this reason many studies do not differentiate between them, and consider both together a slow-oxidative type.

Applying the protocol proposed in the present study to bird muscles with different fiber type compositions,

TABLE 5. Significance levels for total statistical differences in the innervation frequencies between the four fiber types

	I	IIA	IIB	III
I	—			
IIA	**	—		
IIB	**	NS	—	
III	*	**	**	—

NS: Non-significant differences ($P > 0.1$); *$0.05 > P > 0.01$; **$P < 0.01$.

TABLE 6. Innervation frequencies of the different skeletal muscle fiber types as observed in the sampled fields corresponding to the muscles studied

Fiber type	Muscle	n	$\bar{x}$	S^2_{n-1}	Range
I	$SMP_{(p)}$	10	12.28	12.74	8.98–19.53
I	ITC	38	10.12	5.10	6.67–15.79
IIA	PECT	58	2.34	18.14	0–25.0
IIA	$SMP_{(a)}$	23	2.34	6.60	0–8.08
IIA	ITC	38	2.72	2.40	0–6.36
IIB	PECT	58	1.87	6.96	0–13.66
IIB	$SMP_{(a)}$	23	1.89	3.68	0–5.92
IIB	ITC	38	3.26	5.85	0–11.36
IIA and IIB	PECT	58	1.89	7.29	0–14.53
IIA and IIB	$SMP_{(a)}$	23	1.94	3.64	0–5.70
IIA and IIB	ITC	38	2.87	3.09	0–7.19
III	ALD	30	14.09	4.53	11.01–18.75
III	BRC	27	15.21	6.10	11.55–22.46

we have differentiated four fiber types, which are basically in agreement with the classification proposed by Barnard et al. (1982) and recently applied in some studies (Grove et al., 1989; Lalatta-Costerbosa et al., 1990).

The slow-tonic pattern of pigeon ALD fibers is well known, as demonstrated by electrophysiological recordings and the m-ATPase histochemical profile (Hather and Hikida, 1988). This last one shows a characteristic reaction acid stable/alkali stable that has also been found in ALD from chicken (Asiedu and Shafiq, 1972), quail (Alway et al., 1990), and tonic fibers of rat extraocular muscles (Yellin, 1969). The nerve endings from pigeon ALD (Hather and Hikida, 1988), as well as ALD muscles from other birds species (Boesiger, 1968, 1969; Nene, 1977; Ashmore et al., 1978; Rouaud and Toutant, 1982), have proved to be of "en grappe" type and fibers have multiple innervation. Our results are in agreement with these previous reports and demonstrate, on the same slide, pigeon ALD multiple innervated pattern and the peculiar m-ATPase reaction. In addition, we have found a high innervation density and a uniform distribution of nerve endings, which can be correlated with the tonic character of this muscle. For these reasons we assume that fibers present in pigeon ALD are slow-tonic or type III according to Barnard et al. (1982).

We found that pigeon BRC has the same nature as ALD because of the concordance in the m-ATPase reaction (type III fibers), structure of nerve endings, multiple fiber innervation, the large number of nerve endings, and the uniform distribution of them through the whole muscle. These results are in agreement with

those obtained by Nene (1977) for pigeon and fowl BRC. However, like many other authors, her report seems to treat the terms tonic and slow as synonyms. Nevertheless, the conclusions about the functional correlations between ALD and BRC pigeon muscles suggested by Nene (1977) are supported by our results. The functional role of these two muscles is to keep the wing folded, BRC maintaining the antebrachium over the brachium and ALD keeping the brachium close to the body (Nene, 1977).

The pigeon PECT is long known to be composed of two fiber types, named fast glycolytic and fast oxidative glycolytic, respectively (Rosser and George, 1986a,b), according to the classification proposed by Peter et al. (1972). The fast-twitch profile of these fibers is supported by electrophysiological (Dial et al., 1988) and histochemical studies (Simpson, 1979). Our results agree with these previous ones showing IIA and IIB fibers after acidic and alkaline preincubations. In addition, Chinoy and George (1965) reported the presence of "en plaque" nerve endings with a fingerlike structure on singly or focally innervated fibers. Furthermore, this nerve ending structure has also been found in PECT of several species of Galliformes and Passeriformes with some differences in the extent between synaptic gutters (Boesiger, 1968, 1969, 1987). The focal localization of AChE activity was early recognized (Koelle and Friedenwald, 1949), but recently, Trotter et al. (1992) described the macroscopic location of nerve endings in quail PECT, which presents a strong aggregated pattern resulting, after consequent stain, in a striped appearance to unaided eye. Our results support these previous ones and show, in addition, no differences in patterns of innervation between IIA and IIB fibers, which is especially clear in transverse acid preincubated sections. Thus, pigeon PECT is composed entirely of fast fibers since only these are able to reach the contraction frequencies required for this muscle to meet the very high power requirements of flapping flight (Goldspink, 1981; Rosser and George, 1986b).

With the naked eye, two distinct slips arising from different ribs are visible in SMP: an anterior pink slip, arising from second and third ribs, and a posterior one, arising from the fourth rib (Hikida and Bock, 1974; Khan, 1979). Histochemically, all fibers of the anterior slip are fast, and fibers present in the posterior slip are slow (Khan, 1979). Our results show, however, histochemical m-ATPase differences between the slow fibers of the posterior SMP slip and slow fibers present in ALD and BRC. In SMP the slow fibers show the typical reversal reaction after acidic and alkali preincubations (acid stable/alkali labile pattern) whereas, as commented above, ALD and BRC fibers are acid stable/alkali stable. This different slow fiber type fit well into the Barnard et al. classification (1982) as type I. Our results show some additional differences between these type I and III fibers, such as lower innervation frequencies, and a trend to random innervation distribution for type I instead of the uniform one found for type III. On the other hand, differences in innervation structures are also considerable. Whereas type III fibers have the typical "en grappe" nerve endings, type I fibers have these distributed as a series of droplets (Hikida and Bock, 1974). This last pattern of innervation is considered by some authors another form of "en plaque" endplate terminating in small knobs (Hikida and Bock, 1974; Khan, 1979), but there are no definitive results that strongly support this statement. In spite of these differences, one important similarity has been detected: both fibers types are multiply innervated. Finally, type I fibers present a lower level of AChE activity (Buckley and Heaton, 1971) on the edge of the fibers. Because of this, the stain intensity of the nerve ending is lighter in these fibers, and prolongated incubation time (45 instead of 30 minutes) in AChE medium is needed.

In pigeon ITC, a low percentage of slow fibers (type I) are present, intermingled between two types of fast fibers (IIA and IIB). Despite similarities found between type I and II fibers of ITC, PECT, and SMP, some differences can be reported: first, SDH activities from both IIA and IIB fiber types are less extreme (IIA are low to moderate and IIB are moderate to high) than respective fibers from both PECT and anterior SMP; second, innervation distribution tends to be random, being less aggregated than in PECT or anterior SMP. These variations found in innervation distribution in all fiber types present in ITC suggest a clear influence of the particular intermingled fiber distribution in this muscle.

There is much controversy about the existence of slow-twitch (type I) fibers in birds. This avian skeletal muscle fiber type cannot be considered equal to the slow-oxidative mammalian type I, because these fibers are multiple innervated and not oxidative. For this reason, Khan (1979) considers these fibers a new type, naming them "type I white" or "slow-twitch glycolytic." It is not totally clear that these fibers are a true twitch type, as is the case of mammalian ones, although some authors (Bock, 1973; Hikida and Bock, 1974; Hikida, 1987) consider them slow-twitch fibers. On the other hand, others (Morgan and Proske, 1984; Rosser and George, 1986b) show some reserve in accepting these statements, since multiply innervated fibers are not typically action potential driven, which implies that they cannot be classified as a twitch fiber type. At this point, it is interesting to mention the finding reported by Proske and Vaughan (1968), who found two types of multiply innervated fibers in a reptile muscle: one which is not able to propagate action potentials and another which may propagate both graded potentials and twitch contractions.

The present study shows a clear difference in m-ATPase histochemical profile, innervation pattern, and nerve ending structure between slow fibers found in ALD and BRC pigeon muscles (type III) and slow fibers seen in the posterior slip of SMP, and in mixed ITC and SCH muscles (type I). This supports the hypothesis that there are at least two different types of slow fibers in bird muscles: a slow tonic one (type III), as is well documented from electrophysiological recordings in ALD (Hather and Hikida, 1988), and another slow type (type I), which would not be a true slow-twitch fiber as known for mammalian muscles. To determine whether this slow fiber type is a twitch type or not, full electrophysiological recordings should be made in posterior slip of SMP or in skinned fibers from ITC or SCH pigeon muscles. Bock and Hikida (1974) reported that these recordings in SMP have been made

but apparently never published (Morgan and Proske, 1984) and the abstract of Bock (1973) does not show definitive results. Further investigations are therefore required on the electrophysiological properties and their possible correlations with histochemical and structural trends in different muscles and fiber types.

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IV

Capillarity and fibre types in locomotory muscles from wild mallard ducks.
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ORIGINAL PAPER

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Capillarity and fibre types in locomotory muscles of wild mallard ducks (*Anas platyrhynchos*)

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Abstract Six locomotory muscles from wild mallard ducks (*Anas platyrhynchos*) were analysed by histochemical methods. Special care was taken in sample procedure in order to describe the heterogeneity found throughout each muscle. Capillarity and fibre-type distributions were correlated to the functional implications and physiological needs of each muscle. Comparisons between our results and similar previous reports on dabbling and diving ducks are also discussed. Muscles from the leg presented the most heterogeneous fibre-type distributions, which are correlated to the wide range of terrestrial and aquatic locomotory performances shown by these animals. More specialized muscles such as pectoralis, used almost exclusively for flapping flight, had more homogeneous fibre-type distributions, whereas muscles from the wing presented a high proportion of glycolytic fibres probably recruited during non-steady flapping flight. Deep muscle pectoralis zones and parts of the gastrocnemius which are closer to the bone are remarkable for their high capillarity indices and oxidative capacities, which suggests that these parts are recruited during sustained flapping flight and swimming. However, two different strategies for achieving these high oxygen needs are evident, indicating that the fibre cross-sectional area plays an important role in the modulation of the oxygen supply to the muscle cells.

Key words Locomotory muscles · Fibre types · Capillarity · Comparative approach · Mallard duck, *Anas platyrhynchos*

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Abbreviations *AChE* acetylcholinesterase · *cap* · mm^{-2} number of capillaries per square millimeter · *CD* capillary density · *C/F* capillary-to-fibre ratio · *EMR* muscle extensor metacarpialis radialis · *FCSA* fibre cross-sectional area · *FD* fibre density · *FG* fast glycolytic · *FOG* fast oxidative glycolytic · *GLE* muscle gastrocnemius lateralis (pars externa) · *GPDH* α-glycerophosphate dehydrogenase · *ITC* muscle iliotibialis cranialis · *m-ATPase* myofibrillar adenosine triphosphatase · *OFA* oxidative fibre area · *OFN* oxidative fibre number · *PEC* muscle pectoralis · *SCH* muscle scapulohumeralis caudalis · *SDH* succinate dehydrogenase · *SO* slow oxidative · *TSC* muscle scapulotriceps or triceps scapularis

Introduction

Depending on their habitat, birds use various forms of locomotion: diving (using legs or wings for underwater propulsion), swimming, running, and different flying modalities (Butler 1991). The energetic requirements demanded by each kind of locomotion impose a wide range of morphological and physiological adaptative constraints which are noted mainly in cardiovascular, respiratory and musculoskeletal systems (Butler 1991; Maina 1993). Among them, those embracing the muscular system are the most striking due to the great plasticity of muscles and to the predominant role that they play in locomotion. Although most birds have two independent locomotory systems, wings and legs, most of the anatomical and physiological studies on avian musculature have concentrated on the pectoralis muscle.

Pectoralis muscle of most species of flying birds is mainly or exclusively composed of FOG fibres, whose aerobic metabolism relies on a mixture of carbohydrate and fat substrates (Rothe et al. 1987; Butler 1991). However, SO fibres have been found in pectoral

muscles of species that perform gliding or soaring flight (Rosser and George 1986a; Meyers 1993) which is believed to be related to a postural role in holding the wings stationary in an outstretched position against the upward movement of air (Rosser and George 1986a). Finally, FG anaerobic fibres predominate in pectoralis muscle of species that perform short and explosive flights (George and Berger 1966; Kaiser and George 1973); they are present in low percentages in strong fliers where they are understood to be used in take-off or in sudden manoeuvring (Rosser and George 1986b).

Little attention has been paid to flight muscles other than pectoralis. Some authors reported functional implications of some wing muscles (Dial et al. 1991; Dial 1992a, b; Vazquez 1992, 1994) and a few studies on their histochemistry and capillarization have been published (George and Berger 1966; Viscor et al. 1991, 1992; Torrella et al. 1993a).

Avian leg muscles present a heterogeneous distribution of SO, FG and FOG fibres (Suzuki et al. 1985; Turner and Butler 1988; Butler 1991; Duchamp et al. 1992). This uneven distribution is similar to that found in mammals (Armstrong and Laughlin 1985; Suzuki and Tamate 1988) and, due to its regional distribution within the muscle, it is postulated that SO fibres play an important role in maintaining posture (Suzuki and Tamate 1979; Suzuki et al. 1982).

This study describes six muscles of wild mallard ducks (*Anas platyrhynchos*) involved in aerial, terrestrial and aquatic locomotion, taking special care in the selection of the microscopical zones from each sample in order to describe their uneven fibre-type distribution.

Materials and methods

Seven wild mallard ducks (*Anas platyrhynchos*) of either sex with a mean body weight of 1009 ± 81 g ($\bar{x} \pm SEM$) were used for this study. Animals were obtained from *Parc Natural del Delta de l'Ebre* (Tarragona, Spain) during January and February of 1992 and 1993. All of them were captured by hunters, supervised by park guards, under the provision of a scientific collector's permit (no. 5605/92) from the *Direcció General d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya.*

The following six muscles, according to Vanden Berge (1979) nomenclature, were selected for this study: four involved in aerial locomotion (PEC, TSC, SCH and EMR); and two concerned in aquatic and terrestrial locomotion (ITC and GLE). PEC (Fig. 1) arises from the carina and inserts on the deltoid crest of the humerus (George and Berger 1966). This muscle provides the power for the downstroke of the wing during flight by depressing the humerus (Hartman 1961). EMR (Fig. 1) has two distinct bellies with their common origin from the lateral epicondyle of the humerus and its insertion on the extensor process of the carpometacarpus (George and Berger 1966). It extends and stabilizes the wrist during different phases of flapping flight (Dial 1992a). TSC (Fig. 2) arises from the inferolateral surface of the scapula and inserts on the dorsal surface of the ulna (George and Berger 1966) extending and stabilizing the elbow during flapping flight (Dial 1992a). SCH (Fig. 2) arises primarily from the lateral surface of the scapula and inserts on the bicipital crest of the humerus (George and Berger 1966). It is involved in retracting and elevating the humerus, and in rotating the wing during the final half of the downstroke (Dial 1992a). ITC (Fig. 3) is a straplike muscle which arises from the anterior iliac crest and inserts on the patellar ligament (George and Berger 1966) functioning to protract the femur (Cracraft 1971). GLE (Fig. 3) has its origin on the surface proximal to the fibular condyle of the femur and ends on the most lateral part of the tendo achilis (George and Berger 1966), having a predominant role in extending the tarsometatarsus (Cracraft 1971).

Whole muscles were excised from each mallard in the case of ITC, GLE, TSC, EMR and SCH, whereas samples from PEC were selected from the mid-belly of the muscle, taking special care in dissecting out the muscles entirely from the superficial to the deep part.

Histochemical analysis

Before freezing in 2-methylbutane cooled to −160 °C with liquid N_2, muscles were marked in order to determine sample orientation when sectioning in a cryostat (Reichart, Jung) at −20 °C. All transversal sections were obtained from the equatorial muscle sample zone, mounted in 2% gelatinized slides and incubated for 5 min in a buffered fixative (Viscor et al. 1992). Thereafter, the following histochemical assays were performed on serial adjacent sections of 14–20 μm thickness: (1) succinate dehydrogenase, SDH (Nachlas et al. 1957); (2) α-glycerophosphate dehydrogenase, GPDH (Wattenberg and Leong 1960); (3) m-ATPase (Brooke and Kaiser 1970); (4) m-ATPase in order to reveal muscle capillaries (Fouces et al. 1993); (5) Sudan Black B (Chiffelle and Putt 1951); (6) combined m-ATPase and acetylcholinesterase technique, AChE (Torrella et al. 1993b).

Sample procedure

Because muscle fibre type heterogeneity is present in most of the avian muscles (George and Talesara 1961; Suzuki and Tamate 1979), we designed a sampling protocol for each muscle that allowed us to describe whole muscle characteristics throughout the different zones.

In all cases the equatorial transverse sections from each muscle were divided into a grid-like structure throughout two-dimensional axes. First, we determined the major axis and calculated its total length which, depending on the muscle, was divided into 2–14 regular intervals. Subsequently, secondary orthogonal axes transecting the divisions were drawn as lines, the total length of which was also divided into two to four regular intervals. This procedure yielded a grid on each sample from which we selected, as measuring fields, some of the areas centred on the intersections. Figures 1–3 show the fields from which we obtained all the morphometric and histochemical data listed below.

The major axis (dorso-ventral) from PEC samples was divided into 14 divisions, of which we selected the 1st, the 5th, the 9th and the 13th intersections with the medium point of the secondary axes. We thus obtained a transect of fields throughout the muscle (Fig. 1) from its ventral (field 1) to its dorsal (field 4) parts. EMR is a two-belly small muscle of which we chose the medium point from each belly numbered as 1 (dorsal) and 2 (ventral) fields (Fig. 1). The total major axis length from TSC muscle samples was divided into four intervals. The intersections with the medium points of the secondary orthogonal axes were selected as measuring points. Thus, TSC was sampled with a three-field transect throughout its anatomically posterior to anterior zones (Fig. 2). SCH is a large and flat muscle, which was divided into six regular intervals throughout its total major axis length. In this case each of the three secondary axes defined transected the three central intervals of the horizontal axis in two points. From the resulting grid (Fig. 2) we selected the six

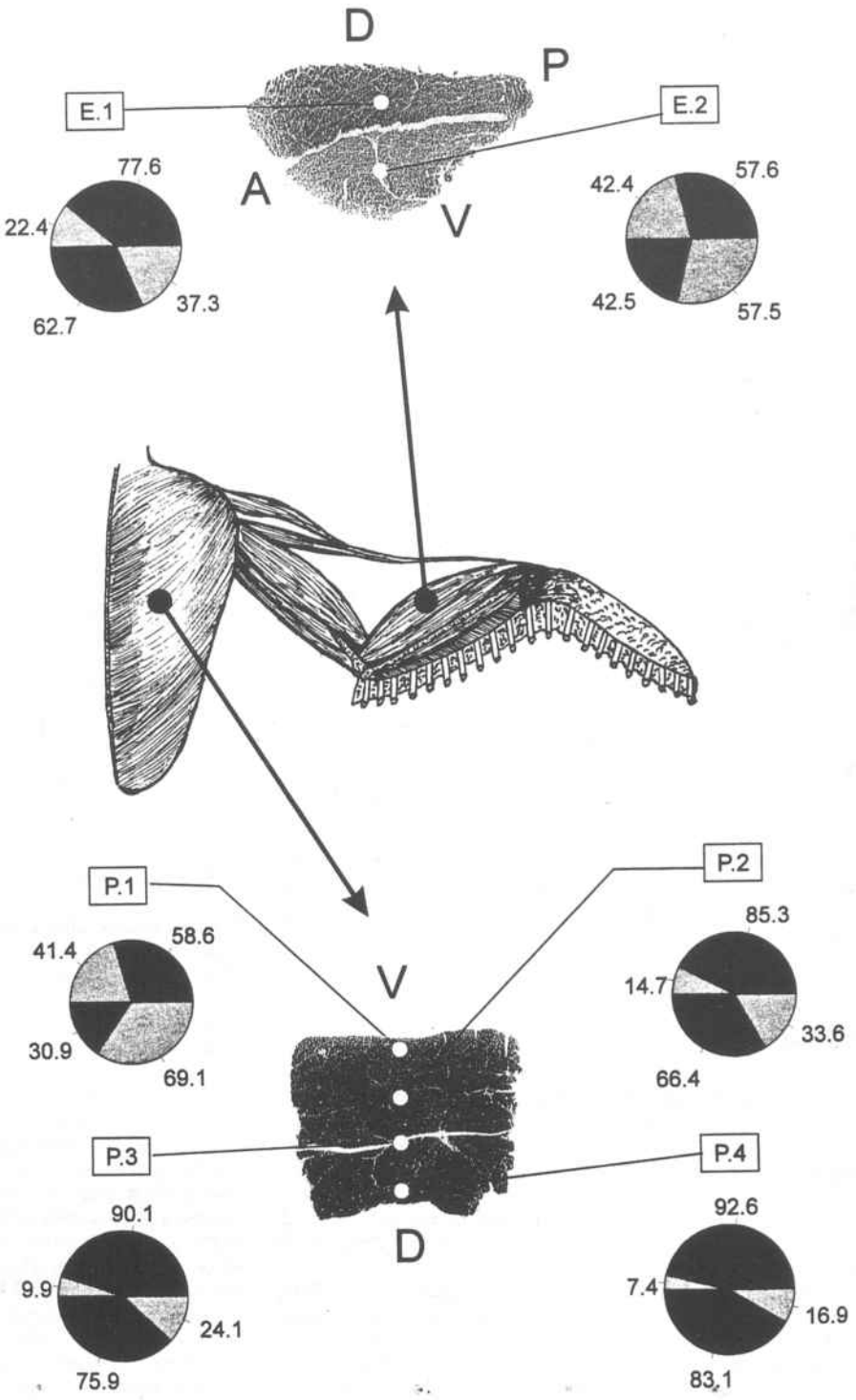

Fig. 1 Ventral view of the mallard wing muscles showing the transverse sections and localization of muscle extensor metacarpialis radialis (EMR, *above*) and muscle pectoralis (PEC, *below*). Each section displays the fields used for fibre typification and capillarization measurements. Numbers are percentage of fibre in number (%FN, *upper semicircles*) and percentage of fibre in area (%FA) occupied by each fibre type (*lower semicircles*). Anatomical localization: *D*, dorsal; *V*, ventral; *A*, anterior; *P*, posterior. Sector code colour: FOG, black; FG, grey

central fields designated from 1 (most posterior and ventral) to 6 (most anterior and dorsal). Four intervals were defined for ITC: from both the first and third of these axis two intersection points with the major axis were selected as measuring fields. These gave a double transect throughout the ITC anatomically internal-external and anterior-posterior parts (Fig. 2). The major GLE axis was divided into four regular intervals which were transected by three secondary orthogonal axes in three points, resulting the grid shown in Fig. 3. Seven measuring points were selected: three in the anterior part of the muscle (fields 1 to 3), one in the middle (field 4) and three more (fields 5 to 7) in the posterior part (Fig. 3).

From the foregoing explanation it is deduced that 26 measuring fields were sampled for this study. These muscle areas will be termed throughout the text, tables and figures using the first initial of the muscle followed by the number of the field (see Figs. 1–3 for the exact localization of each field).

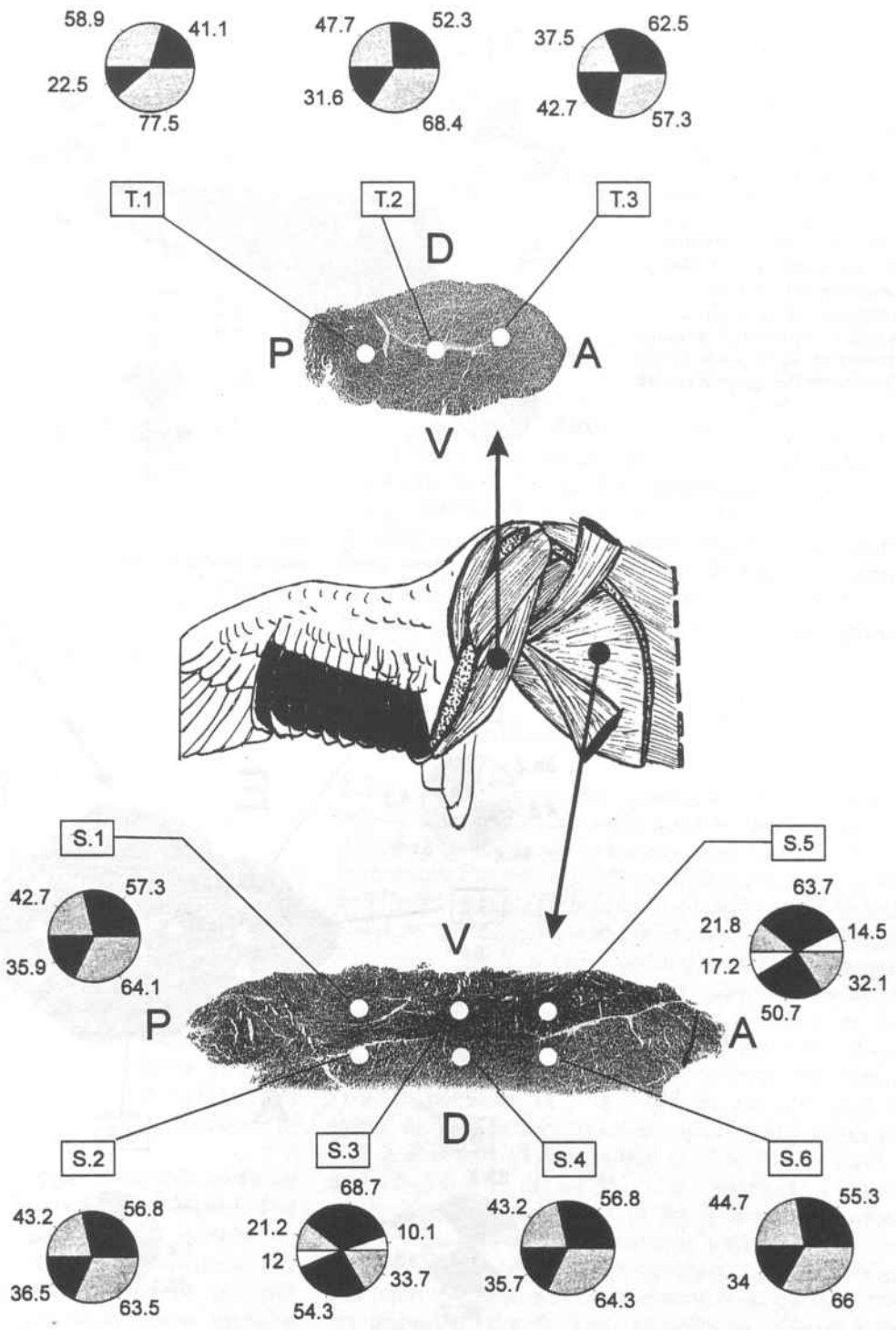

Fig. 2 Dorsal view of the mallard brachium and back muscles showing the transverse sections and localization of muscle triceps scapularis (TSC, *above*) and muscle scapulohumeralis caudalis (SCH, *below*). Each section displays the fields used for fibre typification and capillarization measurements. Numbers are %FN (*upper semicircles*) and %FA occupied by each fibre type (*lower semicircles*). Anatomical localization: *D*, dorsal; *V*, ventral; *A*, anterior; *P*, posterior. Sector code colour: FOG, black; FG, grey; SO, white

Histology

Fibre types were classified according to Peter et al. (1972) using the histochemical assays mentioned above as descriptive criteria. Fibre typification and field measurements were obtained by using a light microscope (Dialux, Leitz, Wetzlar, Germany) equipped with a camera (Wild, MPS 51, Heerburg, Switzerland). Photomicrographs were taken at a magnification of 80x and 200x and fibre measurements were carried out by means of a digitizer tablet (Calcomp 2318-4, Anaheim, Calif., USA) connected to a PC using suitable software (Sigma-Scan, Jandel Scientific, Erkrath, Germany). Photographs of a stage micrometer were taken at the beginning and at the end of each film as calibration control.

CD, FD and C/F were determined from $2 \cdot 10^5$–μm^2 windows in each field and corrected to express them as capillaries and fibres per mm^2. Fibre-type frequencies, in percentage, were obtained by counting at least 100–200 muscle fibres for each field. FCSA of all muscle fibres from each fibre type in all fields were measured in order to find

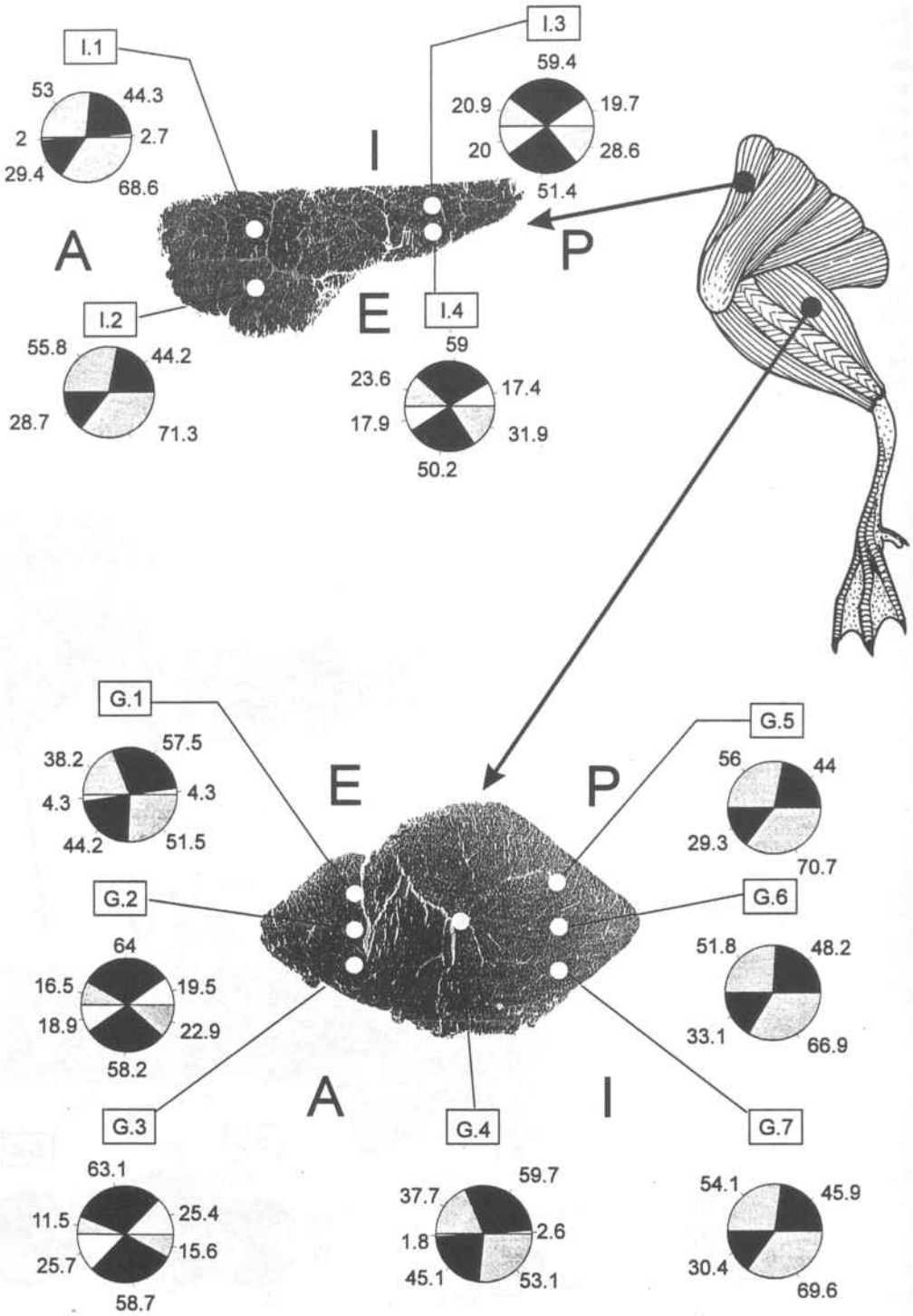

Fig. 3 Lateral view of the mallard leg muscles showing the transverse sections and localization of muscle iliotibialis cranialis (ITC, *above*) and muscle gastrocnemius lateralis (GLE, *below*). Each section displays the fields used for fibre typification and capillarization measurements. Numbers are %FN (*upper semicircles*) and %FA occupied by each fibre type (*lower semicircles*). Anatomical localization: *I*, internal; *E*, external; *A*, anterior; *P*, posterior. Sector code colour: FOG, black; FG, grey; SO, white

the contribution of each fibre type to the total cross-sectional area of the muscle field using the formula:

$$\% FCSA_x = \frac{FCSA_x}{\sum_{i=1}^{n} FCSA_i} \cdot 100$$

Statistics

Data from all variables are expressed as sample means with 95% confidence limits ($\bar{x} \pm t_{\alpha,\sigma} \cdot SEM | \alpha = 0.05, \sigma = 6$, except for fields S.2, S.4 and S.6 where $\sigma = 5$). The variables CD, FD, C/F, %OFN and %OFA (considering SO and FOG together) were analysed using a two-way ANOVA for each muscle taking field and animal as

Table 1 Skeletal muscle fibre types based on their histochemical profile and innervation characteristics

Fiber type	SO	FOG	FG
m-ATPase staining			
alkali preincubation	light	dark	dark
acid preincubation	dark	light	moderate
SDH activity	moderate to high	high	low
GPDH activity	low	moderate to high	high
SUDAN B staining	moderate	dark	light
Innervation pattern	multiple	focal	focal
Neuromuscular junction structure	small knobs	"en plaque"	"en plaque"

factors. A multiple comparison test using Scheffé's procedure was performed in order to determine differences in sample means between fields from the same muscle. Finally, a cluster analysis using the squared Euclidean distance and the complete method was developed. CD, FD, %OFA and percentage of slow fibres area were considered as variables after being standardized.

Results

Fibre types

Table 1 and Fig. 4 show the three fibre types that we have identified in this study by their histochemical properties, innervation pattern and neuromuscular junction structure. In summary, SO fibres had low alkaline and high acidic m-ATPase stability, showed moderate to high SDH and low GPDH staining, and had multiple innervation. Both fast types had high alkaline and low acidic m-ATPase stability and had focal innervation: FG had low SDH and high GPDH staining, whereas FOG had high SDH and moderate to high GPDH staining.

Figures 1–3 show the proportion in number and area of the different fibre types for each field sampled. The three fibre types found (Table 1, Fig. 4) present an uneven distribution throughout all the muscles. FG and FOG fibres are extensively distributed and SO fibres restricted to a few regions of some muscles (Fig. 1–3). Only 9 of the 26 fields studied have slow fibres and 3 of these fields present SO in a lower proportion (both in number and in area) than 5%. This scarce distribution of SO fibres is restricted to two muscles of the leg (GLE and ITC) and one muscle of the wing (SCH). Fields from leg muscles that have more than 10% of SO fibres (G.2, G.3, I.3 and I.4) are in the parts of the muscle closer to the bone (Fig. 3) and those from SCH (S.3 and S.5) are in its anatomically deeper and most anterior part (Fig. 2).

Oxidative parameters

Table 2 shows the oxidative parameters studied for each field: %OFN and %OFA, CD and C/F. It is noted that %OFA from every field are always lower than %OFN, this difference being less marked in the fields that have high %OFN. This is a consequence of the pronounced size differences between FG and FOG fibres, as could be deduced from the FD values (Table 2, Fig. 4). These parameters also show that the most oxidative muscle areas are G.2 and G.3 from GLE, P.3 and P.4 from PEC and I.3 and I.4 from ITC because they have the widest oxidative fibre type distributions and the highest CD. However, C/F does not match these observations as could be deduced from the great apparent differences between these fields. To understand the nature of these differences a plot of the FD versus its CD is displayed in Fig. 5 which shows the relationship between FD and CD which has two extreme values in the most aerobic fields (G.3 and P.4) and marked differences between the slope of the regression lines of some muscles.

Regional muscle variations

Tables 3 and 4 show the significance of the differences between fields and animals, after running a two-way ANOVA test for each parameter and muscle. In order to determine in which fields the differences were present and their level of significance, the results of the Scheffé's multiple comparison test are shown in Figs. 6 and 7. For each muscle a table divided into four triangular sections is presented. Within each table cell the level of significance for FD, CD, C/F and %OFA between all pairs of fields is indicated. Thus, TSC (Fig. 6) shows a clear gradient in %OFA from the anterior (T.3) to posterior (T.1) part of the muscle since the levels of significance between fields vary gradually: $0.05 \geq P > 0.01$ (T.1-T.2), $0.01 \geq P > 0.001$, (T.2-T.3), and $P \leq 0.001$ (T.1-T.3). Less marked gradients throughout the major axis of the muscle are similarly deduced for FD and CD, whereas uniformity is shown for C/F. In the same way, gradients in %OFA, CD and FD from the ventral (P.1) to dorsal (P.4) parts of PEC are apparent (Fig. 6). Ventral fields of SCH (S.3 and S.5) present different levels of significance with the other fields of SCH for %OFA and CD (Fig. 6), indicating the presence of a high aerobic zone in the deep-anterior part of this muscle. ITC presents significant differences in both %OFA and CD between the anterior (I.1, I.2) and posterior (I.3, I.4) fields (Fig. 7). GLE shows also an anterior region (G.2, G.3) with big differences to the other fields for %OFA and CD (Fig. 7). Both C/F and FD showed more homogeneous behaviour within SCH, ITC and GLE (Fig. 6, 7).

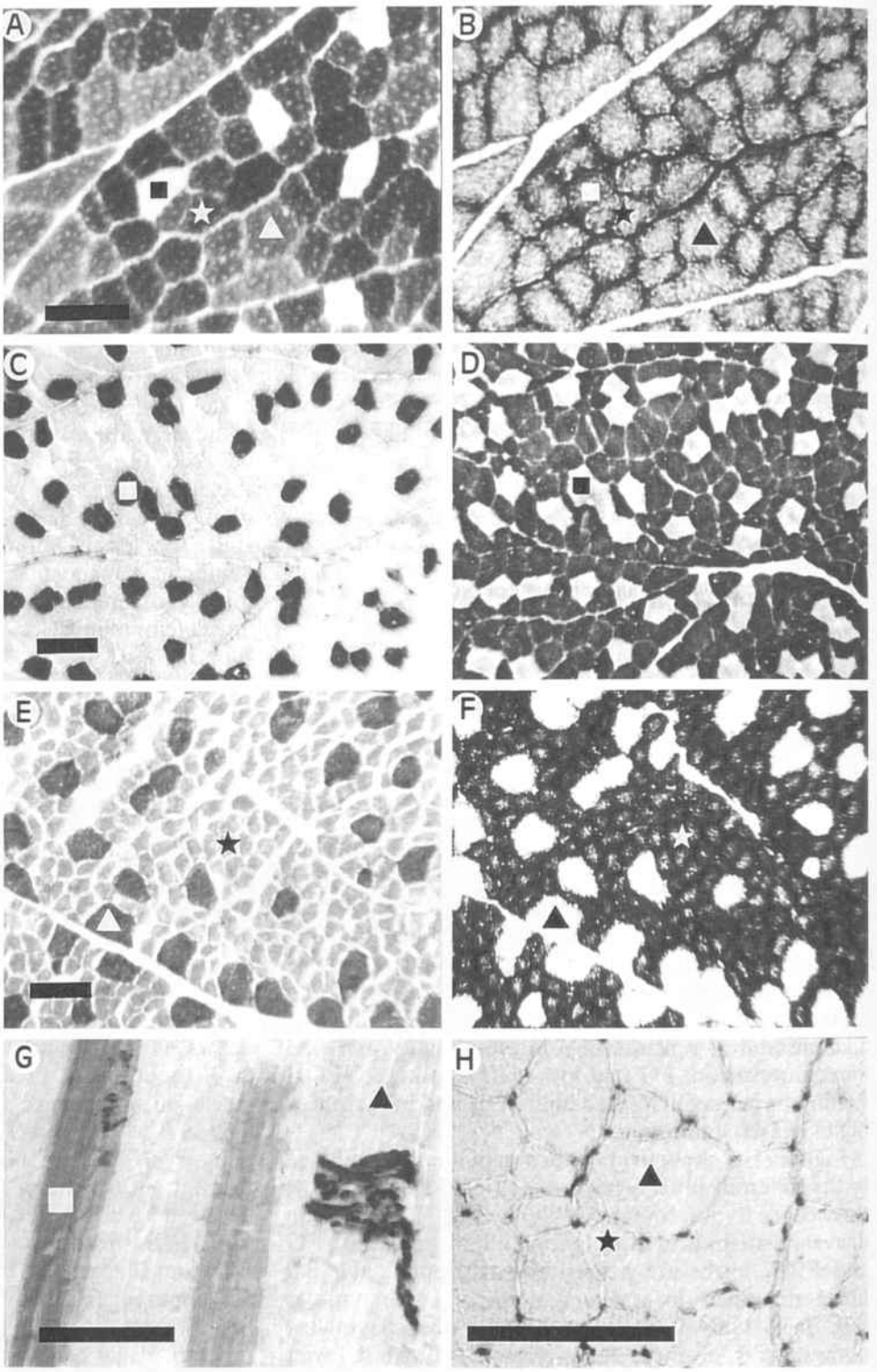

Fig. 4A–H Transverse sections of different mallard muscles (**A**, **B**, **C**, **D**: GLE; **E**, **F**: PEC; **H**: SCH) processed for m-ATPase (**A** and **D** preincubated at pH 11.0; **C** and **E** preincubated at pH 4.2), SDH assay (**B** and **F**), and m-ATPase in order to reveal muscle capillaries (**H**). Micrograph **G** is a longitudinal section of ITC processed for the combined m-ATPase and AChE method preincubated at pH 4.2. Different fibre types are identified on the micrographs. Fibre type codes: SO, *square*; FG, *triangle*; FOG, *star*. *Bar* represents 100 μm

The results of the cluster analysis by the complete method are displayed in Fig. 8. Two groups of fields are segregated at first at a distance $d = 32.0$. On the one hand, there are those fields which have a predominance of anaerobic fibre types, including the superficial zones from PEC and SCH and the posteriorly located fields from GLE, ITC and TSC. Except for E.2 and T.3, for which $d = 5.7$, great similarities are found between all other fields ($d < 2.0$). On the other hand, there are grouped those fields that

Table 2 Oxidative parameters for each field expressed as sample means with 95% confidence limits ($\bar{x} \pm t_{\alpha,\sigma} \cdot SEM$ | $\alpha = 0.05$, $\sigma = 6$, except for fields S.2, S.4 and S.6 where $\sigma = 5$). Fields are named by the first letter of the muscle followed by the number of the field (Figs. 1–3)

Muscle field	%OFN	%OFA	CD	FD	C/F
E.1	77.6 ± 5.8	62.7 ± 8.5	1064.0 ± 100.7	695.9 ± 53.5	1.53 ± 0.11
E.2	57.6 ± 5.9	42.5 ± 6.2	878.3 ± 100.2	596.6 ± 42.4	1.47 ± 0.15
G.1	61.8 ± 11.0	48.5 ± 14.5	871.4 ± 131.3	413.1 ± 57.2	2.13 ± 0.35
G.2	83.5 ± 5.3	77.1 ± 8.3	1229.9 ± 151.0	491.7 ± 92.0	2.55 ± 0.37
G.3	88.5 ± 4.1	84.4 ± 6.3	1384.7 ± 266.8	489.7 ± 80.0	2.89 ± 0.67
G.4	62.3 ± 13.6	46.9 ± 15.1	947.3 ± 83.7	427.1 ± 54.2	2.24 ± 0.24
G.5	44.0 ± 5.7	29.3 ± 5.0	728.0 ± 96.3	385.9 ± 52.9	1.91 ± 0.30
G.6	48.2 ± 10.4	33.1 ± 10.6	760.0 ± 40.0	389.4 ± 43.7	1.98 ± 0.24
G.7	45.9 ± 7.8	30.4 ± 6.4	755.7 ± 71.5	390.1 ± 61.7	1.97 ± 0.28
I.1	47.0 ± 7.3	31.4 ± 6.3	907.6 ± 128.8	532.9 ± 64.4	1.70 ± 0.10
I.2	44.2 ± 6.2	28.7 ± 5.8	807.3 ± 84.4	502.9 ± 75.5	1.63 ± 0.19
I.3	79.1 ± 9.0	71.4 ± 10.9	1230.7 ± 138.0	637.7 ± 73.1	1.94 ± 0.20
I.4	76.4 ± 11.9	68.1 ± 14.9	1168.6 ± 134.4	620.4 ± 71.3	1.89 ± 0.12
P.1	58.6 ± 7.9	30.9 ± 6.7	767.9 ± 139.9	475.0 ± 147.3	1.68 ± 0.24
P.2	85.3 ± 3.3	66.4 ± 6.0	925.3 ± 41.3	717.0 ± 105.4	1.31 ± 0.17
P.3	90.1 ± 1.0	75.9 ± 2.4	1113.4 ± 93.3	793.1 ± 198.0	1.47 ± 0.31
P.4	92.6 ± 2.2	83.1 ± 4.2	1339.4 ± 121.3	930.0 ± 230.4	1.51 ± 0.31
T.1	41.1 ± 4.7	22.5 ± 3.3	814.4 ± 90.5	449.0 ± 53.2	1.91 ± 0.18
T.2	52.3 ± 6.3	31.6 ± 3.8	912.9 ± 94.4	488.4 ± 56.5	1.88 ± 0.20
T.3	62.5 ± 7.4	42.7 ± 8.0	1063.1 ± 89.2	606.1 ± 86.7	1.78 ± 0.20
S.1	57.3 ± 7.8	35.9 ± 7.0	768.0 ± 54.0	461.0 ± 46.9	1.68 ± 0.14
S.2	56.8 ± 14.0	36.5 ± 14.9	716.0 ± 76.6	442.2 ± 86.3	1.65 ± 0.24
S.3	78.8 ± 9.2	66.3 ± 12.0	1065.3 ± 139.5	537.4 ± 57.0	1.99 ± 0.20
S.4	56.8 ± 6.4	35.7 ± 8.5	739.2 ± 119.5	442.3 ± 79.6	1.68 ± 0.09
S.5	78.2 ± 5.9	67.9 ± 8.7	1091.4 ± 113.5	586.6 ± 85.4	1.89 ± 0.25
S.6	55.3 ± 7.5	34.0 ± 8.9	772.7 ± 115.0	491.8 ± 68.5	1.57 ± 0.11

Table 3 Two-way ANOVA test showing the significance of the differences between fields for the oxidative parameters

Muscle	%OFN	%OFA	CD	FD	C/F
EMR	**	***	***	**	NS
GLE	***	***	***	***	***
ITC	***	***	***	**	**
PEC	***	***	***	***	**
TSC	***	***	***	**	NS
SCH	***	***	***	***	**

NS = not significant
* = $0.05 \geq P > 0.01$
** = $0.01 \geq P > 0.001$
*** = $P \leq 0.001$

Table 4 Two-way ANOVA test showing the significance of the differences between animals for the oxidative parameters

Muscle	%OFN	%OFA	CD	FD	C/F
EMR	*	NS	**	NS	**
GLE	**	**	**	***	***
ITC	*	NS	**	**	*
PEC	NS	NS	**	***	***
TSC	*	NS	**	NS	NS
SCH	*	**	***	***	NS

NS = not significant
* = $0.05 \geq P > 0.01$
** = $0.01 \geq P > 0.001$
*** = $P \leq 0.001$

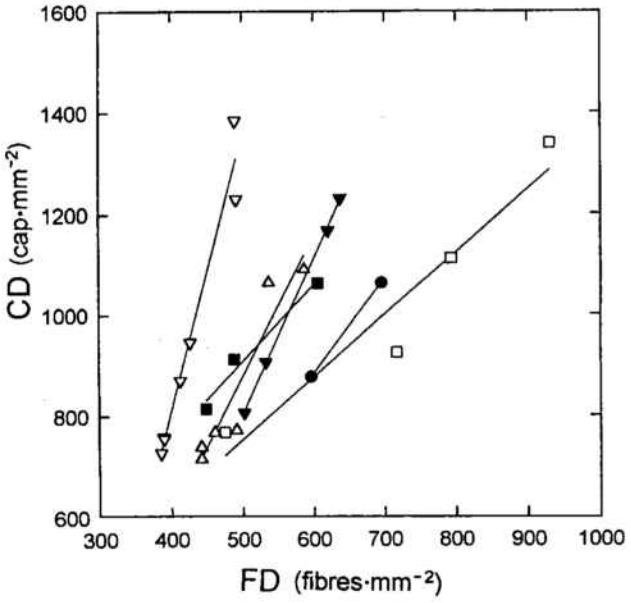

Fig. 5 Relationship between fibre density (*FD*) and capillary density (*CD*) showing the regression lines for each muscle. Each field is represented by a symbol: GLE (▽), SCH (△), PEC (□), TSC (■), EMR (●) and ITC (▼). Each regression line links the fields of the same muscle

rely mainly on oxidative metabolism, which includes the deep portions from PEC and SCH, the dorsal part of EMR and the regions situated close to the bone from GLE and ITC. A second differentiation ($d = 10.8$) between two groups of oxidative fields is also clear: oxidative fields from SCH, ITC and GLE are separated from those of oxidative EMR and PEC. Also remarkable is the segregation of P.4 from P.3 with a value of $d = 7.2$.

Fig. 6 Multiple comparison test by the Scheffé's procedure in fields of muscles involved in aerial locomotion. *Vertical* and *horizontal* rows converge in small cells inside of which the results of the comparison test between the two convergent fields are given: *NS* = not significant; * = $0.05 \geq P > 0.01$; ** = $0.01 \geq P > 0.001$; *** = $P \leq 0.001$

M. Triceps scapularis

FD	3	2	1		1	2	3	% OFA
	**	NS		1		*	***	
	*		NS	2	NS		**	
		NS	NS	3	***	*		
	C/F				CD			

M. Pectoralis

FD	4	3	2	1		1	2	3	4	% OFA
	**	*	NS		1		***	***	***	
	NS	NS		NS	2	NS		*	***	
	NS		NS	NS	3	***	*		NS	
		NS	NS	NS	4	***	***	*		
	C/F					CD				

M. Scapulohumeralis

FD	6	5	4	3	2	1		1	2	3	4	5	6	% OFA
	NS	NS	NS	NS	NS		1		NS	**	NS	***	NS	
	NS	NS	NS	NS		NS	2	NS		**	NS	**	NS	
	NS	NS	NS		NS	NS	3	**	**		**	NS	**	
	NS	NS		NS	NS	NS	4	NS	NS	**		**	NS	
	NS		NS	NS	NS	NS	5	**	***	NS	**		***	
		NS	NS	*	NS	NS	6	NS	NS	**	NS	**		
	C/F							CD						

Discussion

Methodological considerations

The strong significant differences found between the sampled fields (Tables 3, 6, 7) and in some parameters (specially CD) when comparing the same muscles from different animals (Table 4), should be seen as the result of both the muscular tissue heterogeneity and the individual variability of wild mallard ducks. In order to avoid interpretation mistakes, these are two important considerations leading to the conclusion that, specially for low sample sizes, the study of muscular parameters from wild bird populations must take into account both the chosen fields within a muscle and balanced and crossed experimental designs considering individual factors as blocks.

Fibre types and their functional implications

In leg muscles, SO fibres were found in fields situated in muscle regions closer to the bone in approximately 20% of numerical percentage (Fig. 3). It is accepted that these SO fibre-type distribution patterns correlate with a postural role, although a contribution of these fibres to locomotion should not be ruled out. This idea is supported by some reports which show that SO fibres remain active during different types of exercise in mammals (Walmsey et al. 1978; Armstrong and Laughlin 1985). In this sense, Suzuki et al. (1985) suggested that leg muscle SO fibres are recruited during movements and standing in order to maintain the tensions produced by FG and FOG fibres. Patak and Baldwin (1993) have also attributed an important role to slow fibres in emu leg movements, facilitating the elastic

M.Iliotibialis cranialis

	4	3	2	1		1	2	3	4	
	NS	NS	NS		1		NS	***	***	
FD	NS	*		NS	2	NS		***	***	% OFA
	NS		*	NS	3	**	***		NS	
		NS	NS	NS	4	*	***	NS		
		C/F					CD			

M. Gastrocnemius lateralis

	7	6	5	4	3	2	1		1	2	3	4	5	6	7	
	NS	NS	NS	NS	NS	NS		1		**	***	NS	NS	NS	NS	
	NS	NS	NS	NS	NS		NS	2	**		NS	**	***	***	***	
	NS	NS	NS	NS		NS	NS	3	***	NS		***	***	***	***	
FD	NS	NS	NS		NS	NS	NS	4	NS	NS	***		NS	NS	NS	% OFA
	NS	NS		NS	**	NS	NS	5	NS	***	***	NS		NS	NS	
	NS		NS	NS	*	NS	NS	6	NS	***	***	NS	NS		NS	
		NS	NS	NS	*	NS	NS	7	NS	***	***	NS	NS	NS		
			C/F							CD						

Fig. 7 Multiple comparison test by the Scheffé's procedure in fields of the leg muscles. *Vertical* and *horizontal* rows converge in small cells inside of which the results of the comparison test between the two convergent fields are given: *NS* = not significant; * = $0.05 \geq P > 0.01$; ** = $0.01 \geq P > 0.001$; *** = $P \leq 0.001$

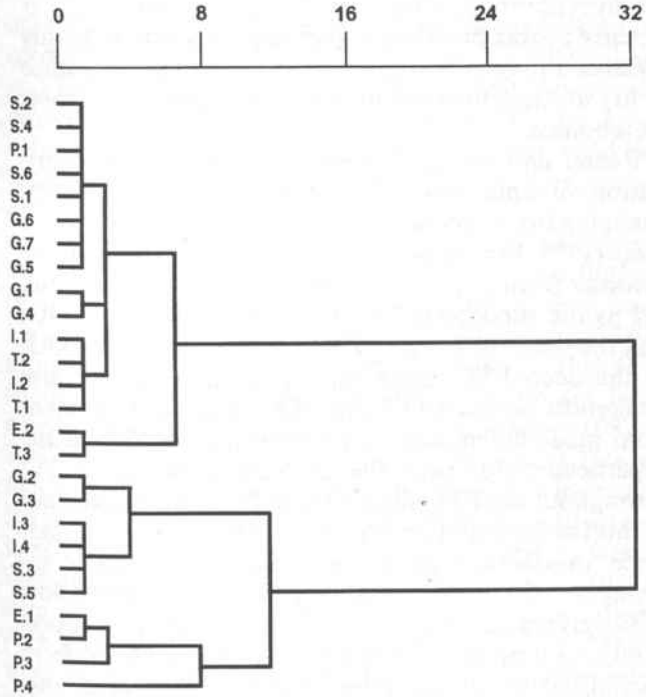

Fig. 8 Cluster analysis of all the fields studied by using the squared Euclidean distance and the complete method. On the *left*, fields are named by the first letter of the muscle followed by the number of the field (Fig. 1–3)

energy storage in the tendons during locomotion. Although mallard ducks are aquatic birds, their legs must also serve for locomotion on land since mallards frequently perform a wide range of activities (preening, oiling or even feeding) out of water (Cramp and Simmons 1977). For these purposes these birds may need relatively high percentages of SO fibres in some regions of their leg muscles in order to efficiently walk and maintain posture on land.

SO fibres are also present in deeper regions of SCH with numerical proportions ranging from 10 to 15% (Fig. 2). Again, a role in posture is attributed to these fibres due to their anatomical localization, in accordance with studies on other vertebrate groups (Rivero et al. 1993; Braund et al. 1995). Rosser and George (1985) reported that the compartimentalization of ostrich pectoralis, in which they found slow- and fast-contracting fibres, is indicative of both slow postural and rapid brief contractions. In a similar way, in mallards the SO fibres present in deep SCH may contribute to maintaining the wing folded by pulling the humerus to the body, although this function is largely carried out by other more specialized muscles such as the anterior latissimus dorsi which has a particular slow tonic fibre type for this purpose (Nene 1977; Torrella et al. 1993a).

The great heterogeneity in fibre-type distributions (Fig. 1–3), the characteristics of the CD versus FD

relationship (Fig. 5) and the gradient exhibited by %OFA and CD throughout the muscle fields (Fig. 6, 7) should be regarded as a consequence of the different types of locomotion in mallards. It is accepted that the contractile properties of any muscle are directly related to its fibre-type composition (Johnston 1985), and that muscles with heterogeneous fibre-type distributions are able to perform a graded recruitment of fibres (Parkhouse 1988; Hermanson et al. 1993). Muscles from the leg (GLE and ITC) are of a type capable of developing a wide range of locomotory activities such as terrestrial locomotion and different swimming intensities. The prevalance of FG fibres in a posterior localization (Fig. 3) seems to be suited to powerful extension of the tarsal (GLE) and knee (ITC) joints when these fibres are recruited for rapid movements. This contrasts with FOG fibres which could be recruited progressively as sustained swimming increases.

PEC has a more homogeneous distribution of fibre types than that observed for leg muscles (Fig. 1). This may be the result of a locomotory performance specialization of this muscle for flapping flight. As in other bird species (Rosser et al. 1987; Turner and Butler 1988; Butler 1991; Viscor et al. 1992), mallard PEC consists predominantly of FOG fibres with a small percentage of FG fibres that changes with muscle depth (Fig. 1). This finding contrasts with other studies on avian pectoralis where a uniform distribution of FOG fibres has been described in species such as pelicans, coots, vultures or cormorants which do not attain rapid ascents during take-off (George and Berger 1966; Rosser and George 1986b; León-Velarde et al. 1993; Rosser et al. 1994). Thus, the regional variation found in mallard pectoralis is interpreted as a functional specialization within the muscle, where FG fibres would be used for short periods of intense activity as in take-off, landing, and in rapid changes in speed and direction during manoeuvring (Rosser and George 1986b; Rosser et al. 1987; Lundgren and Kiessling 1988). Interestingly, Sokoloff et al. (1989) demonstrated an anatomical segregation of the motoneurons innervating superficial and deep parts of the pigeon pectoralis which matched differences in fibre composition and fascicle architecture. Similarities between pigeon and mallard pectoralis at the levels of muscular structure and regional variation tentatively suggest that some degree of segregation in mallard pectoralis innervation could also occur.

According to Dial (1992a) the brachial, antebrachial and shoulder muscles in pigeon are active during each wingbeat cycle in both level and non-steady flapping flight. However, their degree of activity varied greatly between the different modes of non-steady flapping flight and concluded that for this reason these muscles are well developed in birds that perform a substantial amount of these modes of flight (Dial 1992b). Since the recruitment of FG fibres in flight muscles take place during short periods of intense activity, i.e. non-steady flapping flight, the finding of highly anaerobic muscle zones in brachial, antebrachial and shoulder muscles should not be surprising. Thus, FG fibres from SCH dorsal fields (Fig. 2) could be recruited when highly energetic bursts are needed during non-steady flight. The deepest aerobic fields containing FOG fibres would be progressively recruited as level flapping flight develops and when few but more sustained SCH activity is required.

A similar activation pattern is proposed for TSC which has an anterior-posterior gradient of fibre types (Fig. 2, Table 2). In this case the posterior distribution of the anaerobic fibres could give a greater moment arm for stabilizing the elbow joint during the downstroke-upstroke transition.

The situation is slightly different for EMR since FG fibres are not so extensively distributed throughout both EMR muscle bellies (Fig. 1, Table 2). This scarcity of FG fibres could be explained in part by the effect of automating muscular mechanisms during elbow extension that pull the humer to fully extend the manus (Vazquez 1994). These mechanisms could reduce the level of work required by EMR for extending the hand during the upstroke-downstroke transition.

Capillarization and aerobic muscle capacity

Our results show (Table 2) that fields having greater oxidative areas also have higher CD which agrees with previous studies on other species (Schmidt-Nielsen and Pennycuik 1961; Gray et al. 1983). Since CD is linearly proportional to mitochondrial volume density (Weibel 1984), this will provide a continuous source of O_2 to these fields which have high levels of aerobic metabolism.

Rosser and George (1986b) found an uneven distribution of fibre types throughout PEC of mallards, changing from superficial (27% FG and 73% FOG) to deep (13% FG and 87% FOG) muscle zones. If we consider from our data the mean of the fields P.1 and P.2 as the superficial PEC (28% FG and 72% FOG) and the mean of P.3 and P.4 (9% FG and 91% FOG) as the deep PEC, close similarities are evident. Size differences between FG and FOG fibres are apparent from great differences in %OFN and %OFA in the superficial fields and the increasing values of FD throughout the PEC fields (Table 2). As a consequence of this the potential contribution of the FG to the total force production increases rapidly with small increments of the FG frequency (Kaplan and Goslow 1989), giving additional support to the idea that superficial and deep parts of the pectoralis muscle perform specialized functional roles. The relatively great distance between P.4 and P.3 found in the cluster analysis (Fig. 8) could reinforce this statement.

Snyder (1990) reported values of CD = 1014 cap·mm^{-2} and C/F = 1.89 from the aerobic GLE and

CD = 747 cap·mm^{-2} and C/F = 1.16 from the anaerobic GLE. Considering the mean of the fields G.1, G.2 and G.3 as the aerobic region (CD = 1162 cap·mm^{-2} and C/F = 2.52) and the mean of the fields G.5, G.6 and G.7 as the anaerobic GLE (CD = 748 cap·mm^{-2} and C/F = 1.95), our data show great similarities with those obtained by Snyder (1990) for CD but considerably greater values for C/F. This suggests a strong difference in FD and, therefore, in FCSA. Since the mallards used for Snyder's study were obtained from "commercial breeders", whereas those studied here were wild ducks, morphometrical differences in FCSA could be seen as the result of important constraints associated with the process of domestication. Concerning the other leg muscle studied (ITC), our findings also agree with those of Swatland (1985) who reported three fibre types in ITC of domestic ducks having the same SDH and m-ATPase profiles as in our results (Table 1).

Turner and Butler (1988) analysed the fibre-type proportions and capillarity in the locomotory muscles of the tufted duck (*Aythya fuligula*). Their CD results from the oxidative and anaerobic areas of GLE (1313 and 895 cap·mm^{-2}, respectively) are slightly higher than the calculated means from oxidative and anaerobic fields obtained from our study (1162 and 748 cap·mm^{-2}) although, depending on the field considered, this difference is irrelevant (Table 2, field G.3). This slightly greater aerobic capacity of tufted ducks compared to mallard ducks, together with the absence of FG fibres in tufted duck aerobic GLE, may be correlated with physiological differences between diving and dabbling ducks, since aerobic leg propulsion is needed by tufted ducks during feeding dives (Woakes and Butler 1983; Butler 1991). In contrast, fibre-type proportions in the anaerobic GLE are exactly the same in both species (46% FOG and 54% FG); this could be the consequence of similar locomotory performances for this part of the muscle.

Concerning the PEC, although gross morphologies are similar, CD values reported by Turner and Butler (1988) for tufted ducks are 2.5 times greater than our highest CD value for mallards (3361 versus 1339 cap·mm^{-2}). Several factors could account for this difference: it is well known that diving ducks are less buoyant than dabbling ducks (Furilla and Jones 1987). The wing morphology could be an important factor that would contribute to reduce the buoyancy index in the tufted duck, which presents wings with small areas in relation to its body weight. Comparisons between both species calculated from Viscor and Fuster's (1987) data compilation show higher wing loadings in tufted (1.56 g·cm^{-2}) than mallard ducks (1.19 g·cm^{-2}). This wing morphology means greater power requirements during flapping flight (Rayner 1987) which could be satisfied in part by higher CD and smaller fibres, as deduced from C/F (2.20 versus 1.47–1.51). From a physiological point of view, there is another interesting factor that could account for this strong CD difference between both species: hypervolaemia. Keijer and Butler (1982) found that tufted ducks have higher O_2 stores than mallards as a result of a diving adaptative response. They deduced that this difference is in part achieved by means of a greater blood volume, which has also been considered a diving adaptation in other bird species (Bond and Gilbert 1958; Palomeque and Planas 1978; Stephenson et al. 1989). In this sense, the high CD found in tufted ducks could be seen as the consequence of the role played by PEC as an O_2 storage site. This significant venous reserve could be used during diving when peripheral vasoconstriction in PEC blood flow increases the oxygen arterio-venous difference, facilitating the perfusion of active muscles (Stephenson et al. 1986; Butler et al. 1988; Bevan et al. 1992).

Strategies for achieving high oxidative demands

The plot of FD versus CD (Fig. 5) reflects the fact that high oxidative rates may be compatible with either small or relatively large FCSA. The first strategy is that developed in PEC which fits well into the classic profile of highly aerobic muscle: high CD and FD achieved by the small size of its predominant FOG fibres, giving low diffusion distances in order to guarantee an adequate O_2 supply to all the parts of the muscle cells (Banchero 1975; Sullivan and Pittman 1987; Lundgren and Kiessling 1988; Mathieu-Costello 1993; León-Velarde et al. 1993). The second way to reach high oxidative rates would be that shown by the oxidative fields from GLE which must develop high oxidative activity during sustained swimming. These FOG fibres are considerably larger (i.e. low FD; Table 2) than those from PEC. As is well known, the aquatic medium offers higher resistance to movement than air; for this reason, FOG fibres from GLE should be capable of developing not only sustained activity but also high strength contractions. Since large fibres have more contractile units in parallel, they also have a greater capacity for force development (Lucas et al. 1987; Elzinga et al. 1989). Thus, a compromise between the need for power and the ability to fuel sustained muscular work could be achieved by means of FOG fibres with relatively large FCSA. Many physiological variables may minimize the importance of long maximal diffusion distances in those oxidative fibres. Among them, mitochondrial respiration rates, blood transit time or heterogeneity of diffusion along the transport path could play an important role, as proposed by Londraville and Sidell (1990) who also found large oxidative fibres ($>2000\ \mu m^2$) in muscles of Antartic fishes. Moreover, the occurrence of significant radial gradients in SDH activity in fibres of duck and geese muscle (Swatland 1984, 1985) and the evidence that the rate at which the respiratory chain works could be

greater in subsarcolemmal than interfibrillar mitochondrial populations (Philippi and Sillau 1994), reinforce the fact that for achieving high aerobic capacities it is not always necessary to rely on muscle fibres with small size.

On the other hand, the two main categories of muscle fields differentiated by the cluster analyses (Fig. 8), correlate well with the oxidative parameters shown in Table 2 and the physiological and biomechanical properties deduced from our results. Interestingly, the second segregation between the two groups of oxidative fields could be seen as a consequence of the presence of slow fibres. Moreover, the group of fields with slow fibres have a C/F ranging from 1.89 to 2.89, whereas those in which fast fibres are exclusive range from 1.31 to 1.53 (Table 2). This correlation again suggests the existence of two strategies to accomplish high O_2 demands and the important role that the FCSA may play in the modulation of the oxidative capacity of muscles.

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Capillarity and fibre types in locomotory muscles of wild common coots (*Fulica atra*).
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Capillarity and fibre types in locomotory muscles of wild common coots (*Fulica atra*)

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Abstract

Six locomotory muscles of wild common coots (*Fulica atra*) were analyzed histochemically using a sample procedure that describes them extensively. Capillarity and fibre-type distributions were correlated to the functional implications and physiological needs of each muscle. Leg muscles presented three fibre types unevenly distributed, which reflects the great variety of terrestrial and aquatic locomotory performances that coots are able to develop. Aerobic zones are presumably recruited during steady swimming and diving, whilst regions with anaerobic characteristics may be used for bursts of activity such as sprint swimming or during take off, when coots run along the water's surface. Fibre types and capillarization in wing muscles had a marked oxidative trend. High wing beat frequencies, short and broad wings, and the long distance migrations that these birds perform indicate that the presence of high numbers of oxidative fibres and the well developed capillary supply are needed for the great oxygen uptake. Pectoralis muscle, except in its deep part, has exclusively fast oxidative fibres with a very high staining intensity for succinate dehydrogenase assay as compared to the same fibre type of other muscles. Its predominant role in flapping flight justifies these characteristics which are typical of fibres with high aerobic metabolism. The deep part of PEC muscle presents a low proportion of an unusual slow anaerobic fibre type. These fibres could play a role during feeding dives when the bird presses the air out of the feathers by tightening the wings against the body. A linear relationship in all coot muscles studied between CD and FD reflects a perfect adjustment between fibre diameter and vascularization in order to obtain the oxygen for mitochondrial supply. This strategy seems a suitable way to cope with the rigid aerobic constraints that flying and diving impose upon coot's hysiology.

Key words Locomotory muscles · Fibre types · Capillarity · Comparative approach · Common coot, *Fulica atra*

Introduction

An animal's locomotory habits are an important factor in its ecology and physiology. Birds have the ability to develop a wide range of locomotor activities such as swimming, diving, running and, of course, flying (see for review, Butler 1991); which implies the optimization of a species' adaptations to the environment. Since animals must have different fibre types to achieve a full repertoire of movements (Rome 1994), these kind of activities have given rise to a great heterogeneity of the avian muscular tissue and an uneven distribution of fibre types that provide the power to the muscles by means of two forms: anaerobic power, developed in burst activity, and aerobic power, used in sustainable exercise (Rosser and George 1986a; Davis and Guderley 1990).

During flight, birds are capable of producing a great deal of muscular activity, requiring high levels of mechanical and metabolic power (Pennycuick 1975; Rayner 1982). Since flying is an energetically expensive form of locomotion per unit time, birds have been exposed to a strong selective pressure (Tucker 1968; Tobalske and Dial 1994) which is reflected in the various flying styles performed by the different species (Goldspink 1981; Norberg 1985; Rayner 1987; Viscor and Fuster 1987).

Correlations between flying behaviour and the biochemistry and structure of the muscles that power flight have been proved (see George and Berger 1966; Butler 1991). Strong flier birds, such as pigeons or hummingbirds, have pectoralis muscles with high oxidative characteristics. A predominance or an exclusivity of oxidative fibre types (Suarez et al. 1991; Viscor et al. 1992), high mitochondrial volumes and capillary densities (Mathieu-Costello et al. 1994a) and an elevated activity of oxidative enzymes (George and Talesara 1961a,b).

Several groups of birds have adapted independently for life on water, swimming on the surface or diving. These locomotor behaviours are costly in energetic terms, as deduced from the mean oxygen uptake of 3.5-4.1 times that of the resting level (Prange and Schmidt-Nielsen 1970; Woakes and Butler 1983). The fact that the hindlimb muscles of aquatic birds present high levels of regional variation in fibre-type distribution, with segregated red and white areas (Turner and Butler 1988; Torrella et al. 1996), together with the high activities for both oxidative and anaerobic enzymes (Bishop et al. 1995), means that leg muscles of aquatic birds are suitable for both sustained and short burst swimming.

There are species of flightless birds which rely exclusively on their legs for locomotion. In the emu, during running, high aerobic capacity is evident owing to the 11.4 increment of the oxygen consumption above the resting level (Grubb et al. 1983). To attain this, the leg limb muscles of running birds have a greater mass relative to body size than in other birds (Patak and Baldwin 1993). Gastrocnemius muscle of emus does not possess slow fibres, indicating that it has a reduced postural role and the exclusive presence of fast fibres is seen as an specialization to power output during locomotion (Patak and Baldwin 1993).

Apart from locomotory functions, bird muscles are also capable of developing activities which involve low but long-lasting levels of work, such as postural roles of the legs (Suzuki and Tamate 1979; Suzuki et al. 1982; 1985; Boesiger 1991), the maintenance of the wings folded close to the body (Nene 1977; Hikida and Wang 1981; Torrella et al. 1993a), or outstreched during gliding or soaring flight (Goldspink 1981; Rosser and George 1986b; Meyers 1993; Rosser et al. 1994).

Six locomotory muscles of the common coot (*Fulica atra*), a species that can develop aerial, aquatic and terrestrial locomotion, have been selected for this study. The aim of this work is to obtain descriptive and comparative data on fiber types and capillary supply in order to have a better understanding of the ecophysiological adaptation of this species.

Materials and methods

Animals

Six wild common coots (*Fulica atra*) of either sex with a mean body weight of 794 ± 53 grams (mean ± SEM) were used for this study. Animals were obtained from *Parc Natural del Delta de l'Ebre* (Tarragona, Spain) during January and December of 1993. All of them were captured by hunters, supervised by Park guards, under the provision of a scientific collector's permit (no. 5605/92) from the *Direcció General d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya.*

Muscles

The following six muscles, according to Vanden Berge (1979) nomenclature, were selected for this study. Four of them involved in aerial locomotion: muscle pectoralis (PEC), muscle scapulotriceps or triceps scapularis (TSC), muscle scapulohumeralis caudalis (SCH) and muscle extensor matacarpi radialis (EMR); and two concerned with aquatic and terrestrial locomotion: muscle iliotibialis cranialis (ITC) and muscle gastrocnemius lateralis, pars externa (GLE). See figure 1 for the exact location of the muscles.

PEC arises from the ventrolateral surface of the carina and attaches by dorsal fleshy fibres on the deltoid crest of the humerus (George and Berger 1966; Rosser 1980). This muscle provides the power for the downstroke of the wing

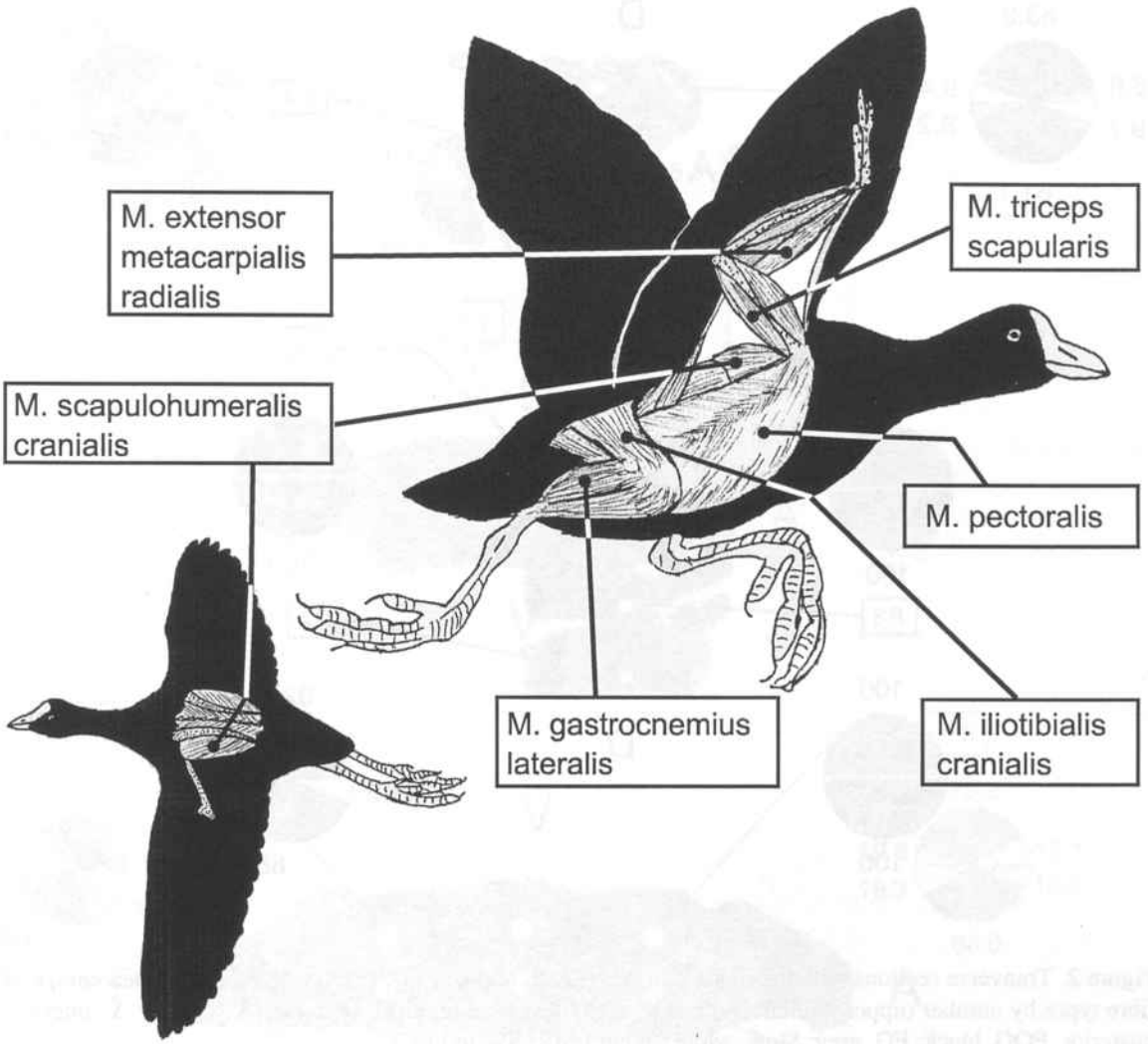

Figure 1. Location of the muscles PEC, EMR, TSC, ITC, SCH and GLE from a lateral view and SCH from a dorsal view.

during flight by depressing the humerus (Hartman 1961). EMR is a fusiform muscle situated on the dorsal edge of the antebrachium and consists of two distinct bellies (Caput ventrale and Caput dorsale). Both bellies arise from a tubercle on the distal dorsal surface of the humerus and fuse near the elbow joint to attach by a common tendon on the extensor process of the carpometacarpus (George and Berger 1966; Rosser 1980). It extends and stabilizes the wrist during different phases of flapping flight (Dial 1992). TSC is a long muscle with its belly on the dorsocaudal surface of the humerus. It arises from the cranial ventrolateral tip of the scapula and inserts by a tendon that attaches to the dorsoproximal tip of the ulna (Rosser 1980). It extends and stabilizes the elbow during flapping flight (Dial 1992). SCH is a large triangular muscle which arises by dorsal fleshy fibres from the lateral surface of the scapula and inserts by tendinous and fleshy fibres on the ventral edge of the humerus (Rosser 1980). It is involved in retracting and elevating the humerus, and in rotating the wing during the final half of the downstroke (Dial 1992). ITC is a straplike muscle which arises from the anterior iliac crest and inserts on the patellar ligament (George and Berger 1966) its function being to protract the femur (Cracraft 1971). GLE has its origin on the surface

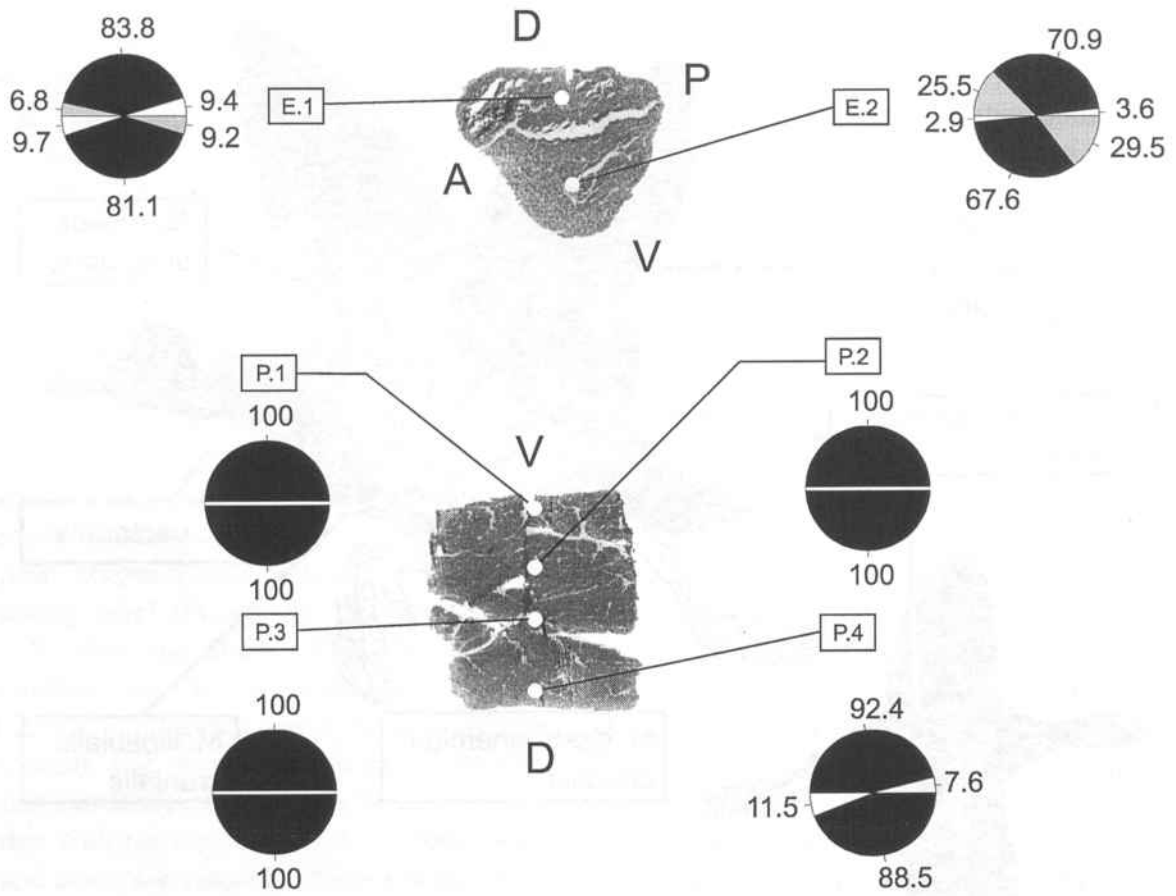

Figure 2. Tranverse sections with the fields sampled (EMR, above; PEC, below). Numbers are percentage of fibre types by number (upper semicircles) and by area (lower semicircles). **D**, dorsal; **V**, ventral; **A**, anterior; **P**, posterior. FOG, black; FG, grey; Slow, white (SO in EMR; SW in PEC).

proximal to the fibular condyle of the femur and ends on the most lateral part of the Tendo Achilis (George and Berger 1966) having a predominant role in extending the tarsometatarsus (Cracraft 1971).

The whole muscles were excised from each coot in the case of ITC, GLE, TSC, EMR and SCH, whereas samples from PEC were selected from the mid belly of the muscle. Special care in dissecting out the entire muscles, from the superficial to the deep part, was taken.

Muscle preparation and histochemistry

Muscle samples were taken by autopsy, frozen and stored in 2-methylbutane, cooled with liquid nitrogen to -160°C, until sectioning in a cryostat (Reichart, Jung) at -20°C. 14-20 µm transverse muscle sections from the equatorial zone were cut serially and collected on gelatinized glass coverslips. Sections were incubated for 5 minutes in a buffered fixative (Viscor et al. 1992) and stained for the following histochemical assays in order to identify the fibre types and capillaries: 1. Succinate dehydrogenase, SDH (Nachlas et al. 1957). 2. α-glycerophosphate dehydrogenase, GPDH (Wattenberg and Leong 1960). 3. myofibrillar adenosine triphosphatase, mATPase (Brooke and Kaiser 1970). 4. ATPase in order to reveal muscle capillaries (Fouces et al. 1993). 5. Sudan Black B (Chiffelle and Putt 1951). 6. Combined mATPase and acetylcholinesterase technique, AChE (Torrella et al. 1993b).

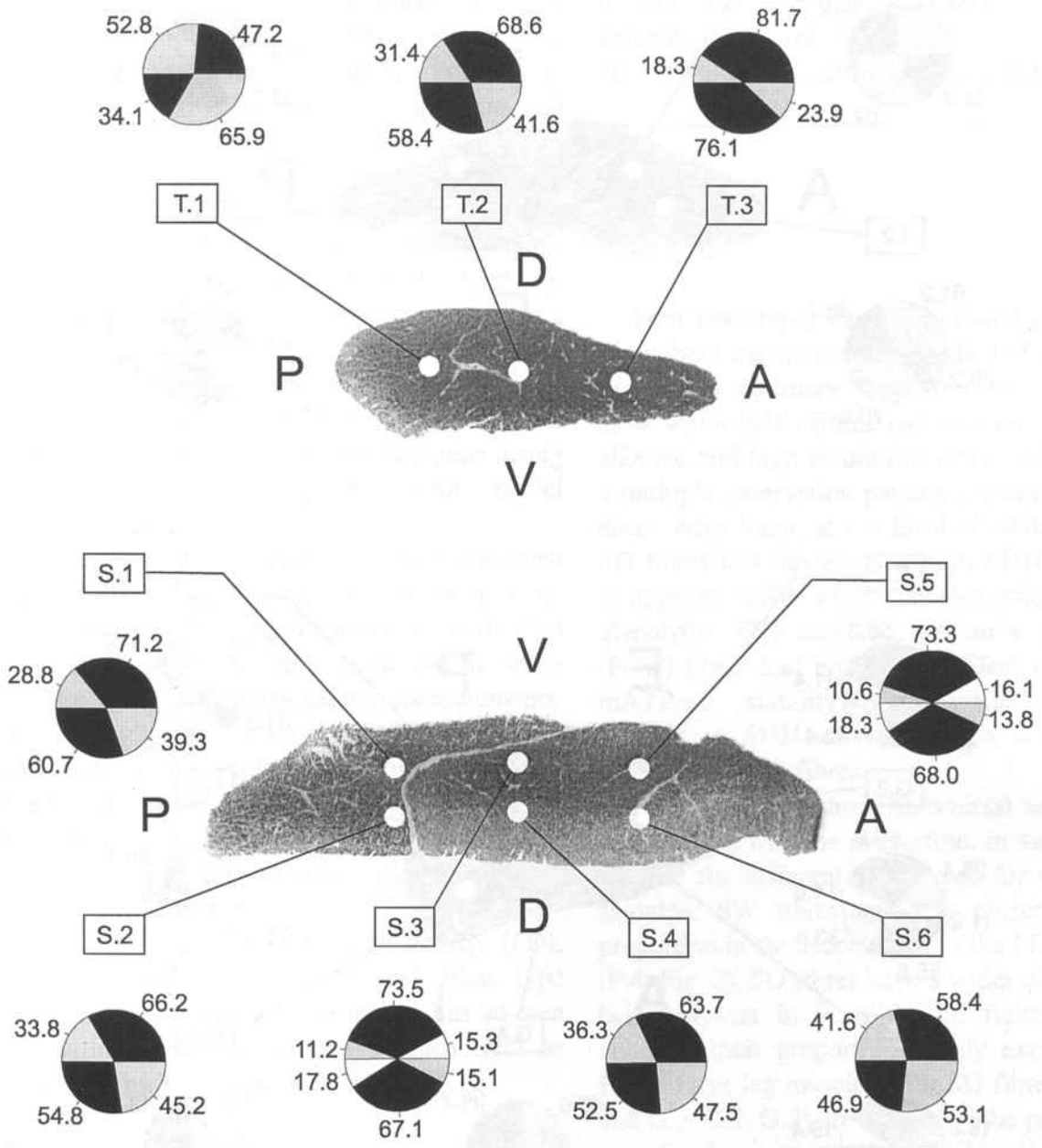

Figure 3. Transverse sections with the fields sampled (TSC, above; SCH, below). Numbers are percentage of fibre types by number (upper semicircles) and by area (lower semicircles). **D**, dorsal; **V**, ventral; **A**, anterior; **P**, posterior; FOG, black; FG, grey; SO, white.

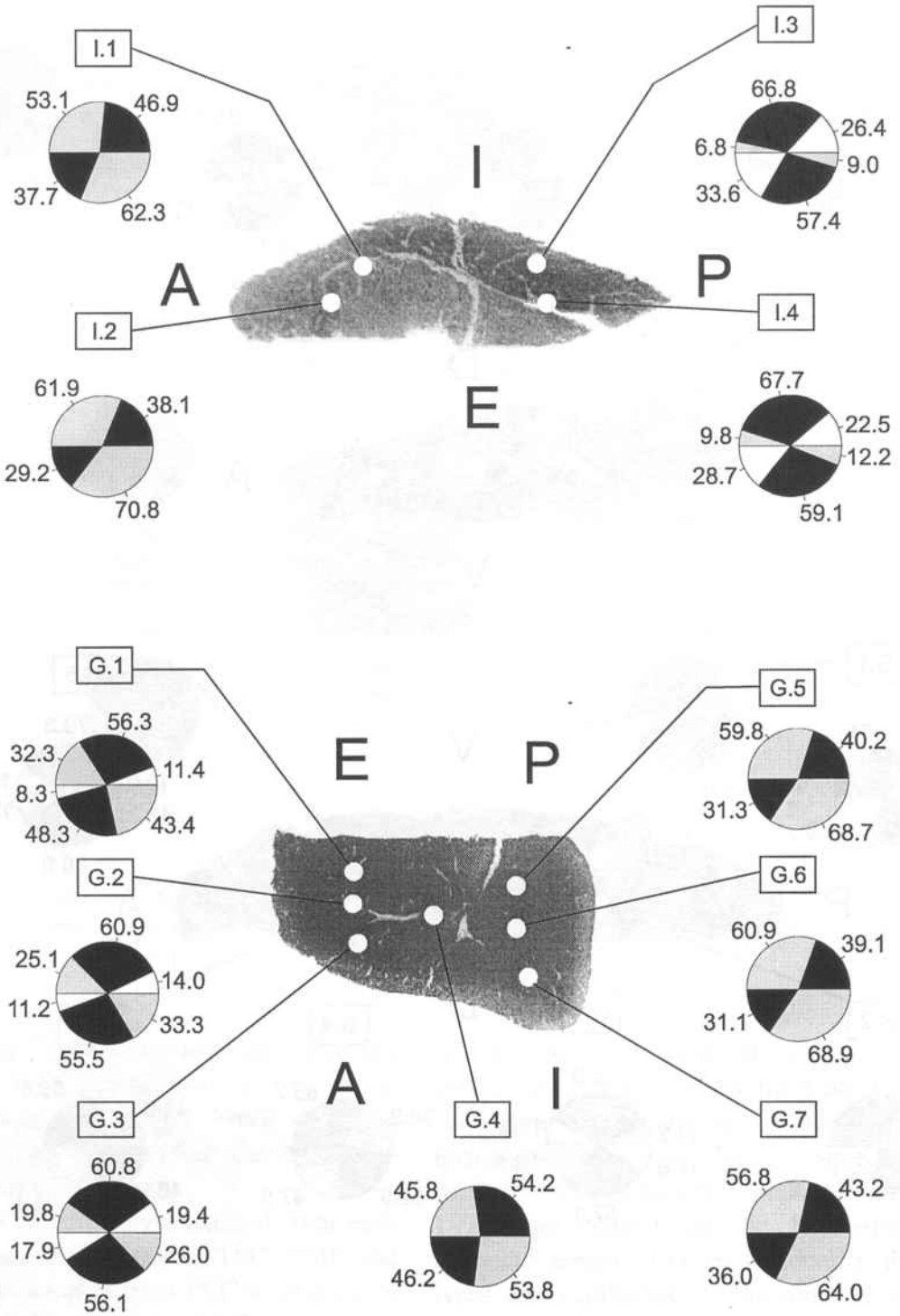

Figure 4. Transverse sections with the fields sampled (ITC, above; GLE, below). Numbers are percentage of fibre types by numbers (upper semicircles) and by area (lower semicircles). **A**, anterior; **P**, posterior; **E**, external; **I**, internal. FOG, black; FG, grey; SO, white.

Tissue analysis

Fibre types were classified according to the basic scheme of Barnard et al. (1982) for bird skeletal muscle, using the histochemical assays mentioned above as descriptive criteria. We have adopted the nomenclature of Peter et al. (1972) because it reflects the metabolic and functional implications of muscle fibres (see Butler 1991). Fibre typification and field measurements were obtained by using a light microscope (Dialux, Leitz, Wetzlar, Germany) equipped with a camera (Wild, MPS51, Heerburg, Switzerland). Photomicrographs were taken at a magnification of x80 and x200 and fibre measurements were carried out by means of a digitizer tablet (Calcomp 2318-4, Anaheim, CA, USA) connected to a personal computer using the suitable software (Sigma Scan, Jandel Scientific, Erkrath, Germany).

By means of the sample procedure designed by Torrella et al. (1996), the equatorial transverse sections from each muscle were divided into a grid-like structure from which some muscle fields were selected for measurements. As a result of this protocol 26 fields were sampled for this study (see Figures 2 to 4 for their exact location). These fields will be termed throughout the text, tables and figures using the first initial of the muscle followed by the number of the field.

Capillary density (CD), fibre density (FD), capillary-to-fibre ratio (C/F) and fibre type frequencies (both as a numerical and as an area proportion) were determined in each field as described by Torrella et al. (1996).

Statistics

Data from all variables are expressed as sample means with 95% confidence limits (mean $\pm$ $t_{\alpha,\sigma}$ | α=0.05, σ=5). The variables CD, FD, C/F, percentage of oxidative fibre number (%OFN, considering slow oxidative and fast oxidative fibres together) and percentage of oxidative fibre area (%OFA) were analyzed using a two-way ANOVA for each muscle taking "field" and "animal" as factors. A multiple comparison test using Scheffé's procedure was performed in order to determine differences in sample means between fields from the same muscle. Finally, a cluster analysis using the squared Euclidean distance and the complete method was developed. CD, FD, %OFA and percentage of slow fibres by area were considered as variables after being standardized.

Results

Fibre types

Four fibre types have been found distributed throughout the six muscles studied (Table 1 and Fig. 5). In summary, slow oxidative (SO) and slow white (SW) fibres had in common a low alkaline and high acidic mATPase stability and a multiple innervation pattern. However, differences were found at the level of SDH activity: SO fibres had moderate to high SDH stainings as opposed to SW which had low stainings. Fast glycolytic (FG) and fast oxidative glycolytic (FOG) fibres had high alkaline and low acidic mATPase stability and presented focal innervation. SDH activity was low in FG fibres and high in FOG fibres.

Figures 2 to 4 show transversal sections of each muscle with the proportion, in number and area, of the different fibre types for each field sampled. SW fibres are only present in low proportion in the deepest part of the PEC muscle (P.4, Fig. 2). SO fibres have a wider distribution being present in 9 of the 26 fields studied, although their proportions rarely exceed 20%. Fields from leg muscles with SO fibres (I.3, I.4 and G.1, G.2, G.3) are located in the parts of the muscle closer to the bone (Fig. 4), whereas fields from SCH (S.3 and S.5) are found in the deepest and most anterior part of the muscle (Fig. 3). FG fibres are widely present over all the fields sampled except in muscle PEC where they are absent. They are distributed in proportions that vary greatly: from less than 10% in the posterior part of ITC and dorsal belly of EMR to more than 50% in the anterior region of ITC and in the posterior GLE (Fig. 4). FOG fibres also have a wide and varied distribution among all the fields of the six muscles studied. Notable,

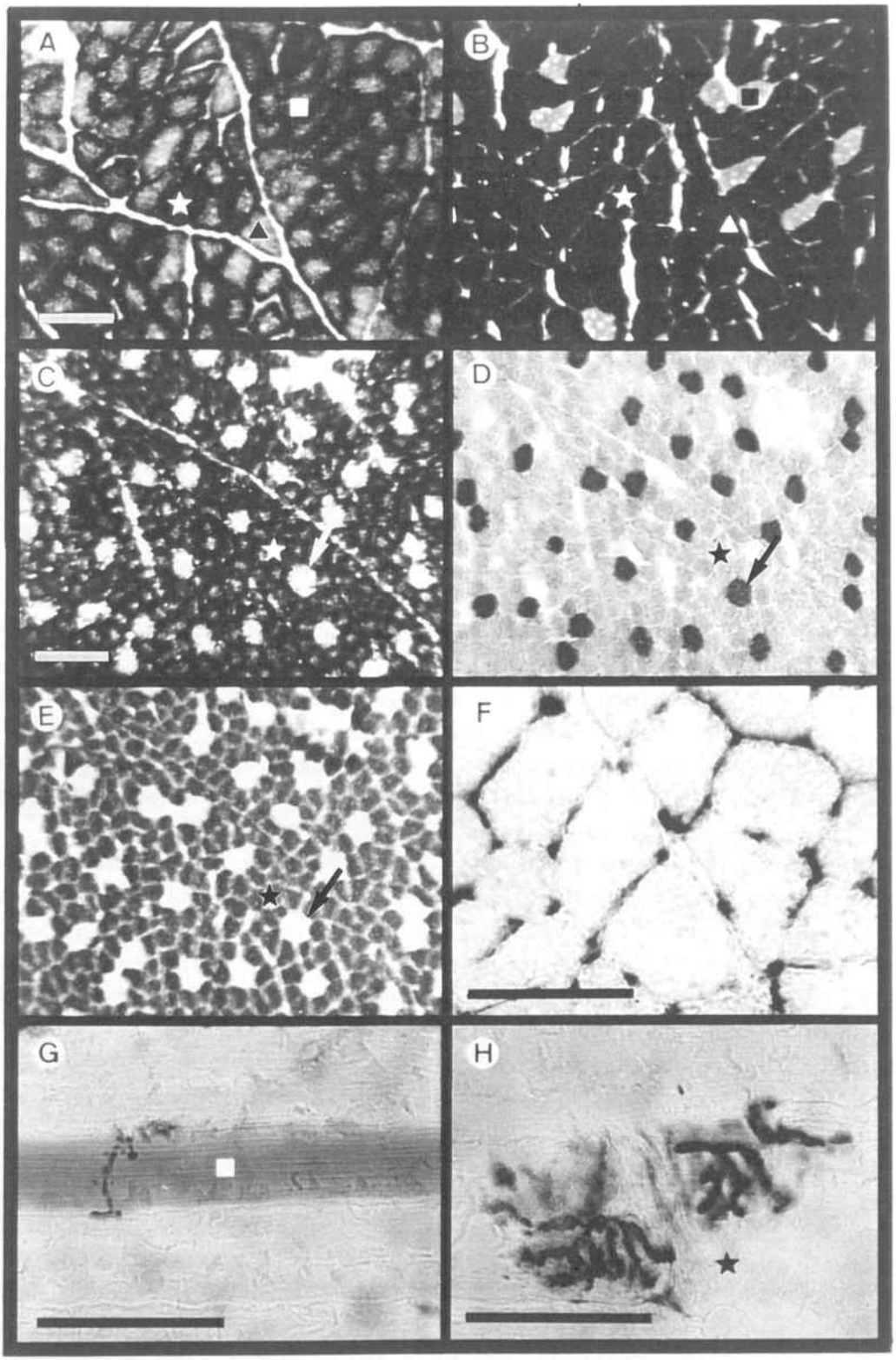

Figure 5. Transverse sections of different coot muscles (**A**, **B**: EMR; **C**, **D**, **E**: deep PEC; **F**: GLE) processed for mATPase (**B** and **E** preincubated at pH 11.0; **D** preincubated at pH 4.2), SDH assay (**A** and **C**), and ATPase in order to reveal muscle capillaries (**F**). Micrographs **G** and **H** are longitudinal sections of ITC processed for the combined mATPase and AChE method preincubated at pH 4.2. Different fibre types are identified on the micropraphs. Fibre type codes : SO, square; FG, triangle; FOG, star; SW, arrow. Bar represents 100 µm.

Table 1. Skeletal muscle fibre types based on their histochemical profile and innervation characteristics.

Fibre type	SO	SW*	FOG	FG
mATPase				
alkali preincubation	light	light	dark	dark
acid preincubation	dark	dark	light	moderate
SDH activity	moderate to high	low	high	low
GPDH activity	low	moderate	moderate to high	high
Sudan B staining	moderate	light	dark	light
Innervation pattern	multiple	multiple	focal	focal
Neuromuscular junction structure	small knobs	small knobs	"en plaque"	"en plaque"

* Only present in the deepest area of muscle pectoralis (P.4)

their presence is exclusive in much of the PEC muscle where their SDH activity is, in particular, very high (Fig. 5C).

Tissue morphological parameters

Table 2 shows the tissue morphological parameters studied for each field: %OFN, %OFA, CD, FD and C/F. The effect of fibre cross-sectional area (FCSA) relative to each fibre type is clearly shown in the %OFN and %OFA values: in all fields where FG fibres are present a difference between these two parameters is noticeable. This difference is more marked in fields that have lower %OFN, which indicates a size difference between FG and the oxidative fibres (Fig. 5A). The oxidative fields (those with high %OFN and %OFA) also have the highest CD, which is especially clear for all PEC muscle. However, due to the very high FD in PEC fields, the C/F values are low in this muscle as compared to the oxidative fields from other muscles. The relationship between FD *versus* CD plotted in Figure 6 shows that changes in FD are paired with changes in CD in a similar way for all the fields sampled. This gives regression lines with similar slopes in muscles where heterogeneity in FD and CD is present.

Muscle and individual variability

The results of the two-way ANOVA test (Table 3) show great significant differences between the fields studied for most of the tissue morphological parameters. Variability was also clear between animals except for the percentages of oxidative fibres, where no significant differences could be detected in any muscle other than TSC (Table 4). Figures 7 and 8 show Scheffé's multiple comparison tests which were used in order to clarify in which pairs of fields the differences were found. The lack of significant differences between almost all fields from all muscles for both FD and C/F is noteworthy. In contrast, %OFA and CD presented significant differences between some fields showing a clear gradient within the same muscle. The most marked gradients were found in SCH, from its dorsal to ventral zones (Figs. 3 and 7); in PEC, where the population of SW fibres in the deep part of the muscle contrasts with the

Table 2. Tissue morphological parameters for each field expressed as sample means with 95% confidence limits. Fields are named by the first letter of the muscle followed by the number of the field (Figures 2-4).

Field	% OFN	% OFA	CD	FD	C/F
E.1	93.2 ± 3.5	90.8 ± 4.8	1660 ± 180.3	732 ± 168.9	2.33 ± 0.41
E.2	74.5 ± 6.2	70.5 ± 6.3	1326 ± 168.7	546 ± 77.9	2.44 ± 0.24
G.1	67.7 ± 6.6	56.7 ± 7.2	1090 ± 140.1	498 ± 92.3	2.23 ± 0.39
G.2	74.8 ± 7.1	66.7 ± 8.3	1159 ± 111.0	503 ± 116.6	2.36 ± 0.35
G.3	80.2 ± 6.8	74.0 ± 8.8	1091 ± 125.0	479 ± 82.5	2.31 ± 0.32
G.4	54.2 ± 12.5	46.2 ± 12.7	936 ± 56.8	436 ± 63.1	2.18 ± 0.32
G.5	40.2 ± 8.4	31.3 ± 6.6	824 ± 81.1	405 ± 84.5	2.07 ± 0.27
G.6	39.1 ± 5.0	31.1 ± 3.5	817 ± 72.1	417 ± 45.2	1.97 ± 0.19
G.7	43.2 ± 15.0	36.0 ± 13.6	807 ± 86.5	423 ± 70.5	1.93 ± 0.25
I.1	46.9 ± 12.6	37.7 ± 13.1	1109 ± 136.5	550 ± 60.6	2.02 ± 0.10
I.2	38.1 ± 3.4	29.2 ± 4.8	1046 ± 190.9	555 ± 53.1	1.89 ± 0.31
I.3	93.3 ± 4.0	91.0 ± 5.4	1450 ± 139.8	716 ± 104.5	2.05 ± 0.32
I.4	90.3 ± 8.6	87.8 ± 9.5	1455 ± 261.5	686 ± 111.2	2.13 ± 0.32
P.1	100.0 ± 0.0	100.0 ± 0.0	1579 ± 168.5	832 ± 75.8	1.90 ± 0.11
P.2	100.0 ± 0.0	100.0 ± 0.0	1571 ± 183.6	861 ± 133.5	1.84 ± 0.20
P.3	100.0 ± 0.0	100.0 ± 0.0	1539 ± 236.4	829 ± 90.0	1.86 ± 0.23
P.4	92.4 ± 2.3	88.5 ± 3.0	1541 ± 319.8	859 ± 167.8	1.79 ± 0.14
T.1	47.2 ± 7.4	34.1 ± 6.8	1042 ± 183.4	510 ± 98.8	2.06 ± 0.19
T.2	68.6 ± 10.2	58.4 ± 12.8	1215 ± 153.1	593 ± 90.9	2.07 ± 0.30
T.3	81.7 ± 6.1	76.1 ± 7.7	1371 ± 173.0	678 ± 123.2	2.05 ± 0.27
S.1	71.2 ± 4.8	60.7 ± 6.2	1028 ± 185.8	491 ± 100.3	2.10 ± 0.12
S.2	66.2 ± 8.3	54.8 ± 10.4	925 ± 112.5	453 ± 36.9	2.04 ± 0.16
S.3	88.8 ± 5.8	84.9 ± 8.1	1229 ± 212.5	592 ± 88.2	2.09 ± 0.27
S.4	63.7 ± 7.4	52.5 ± 8.4	1002 ± 174.3	532 ± 82.7	1.88 ± 0.13
S.5	89.4 ± 4.4	86.2 ± 5.8	1259 ± 201.9	583 ± 80.0	2.16 ± 0.13
S.6	58.4 ± 15.1	46.9 ± 15.5	1001 ± 224.5	546 ± 107.2	1.83 ± 0.10

homogeneity of the rest (Figs. 2 and 7); and in GLE from its anterior to posterior parts (Figs. 4 and 8). A slight gradient for CD from the internal to external ITC was also noted (Fig. 8).

Figure 9 shows the results of the cluster analysis by the complete method. Two groups of fields, separated at a distance d=28.8, are evident at first sight. A highly oxidative group, having more than 1,450 cap/mm^2 and %OFN over 90% (Table 2), is formed by the four PEC fields, the internal ITC (I.3 and I.4) and the dorsal part of EMR (E.1). A second group consists of two populations of fields, segregated at d=13.2. On the one hand is a clearly anaerobic population of fields, which rarely surpasses 50% of %OFA (Table 2), integrated by the posterior GLE (G.4-G.7), the postero-dorsal SCH (S.1, S.2, S.4, S.6), the anterior ITC (I.1, I.2) and the posterior TSC (T.1). On the other hand, there is an aerobic group with intermediate values of CD, ranging from 1,000 to 1,400 cap/mm^2, and %OFN from 60% to 90% (Table 2); formed by the anterior GLE (G.1-G.3), the anterior TSC (T2, T.3), the ventral EMR (E.2) and the ventral SCH (S.3, S.5). Interestingly, slow fibres are only found in the "aerobic" groups, where the fields containing significative amounts of these fibres are segregated apart from the rest of the subgroup (branches at d=13.4 and d=6.5 in Fig. 9).

Table 3. Two-way ANOVA test showing the significance of the differences between fields for the tissue morphological parameters.

Muscle	%OFN	%OFA	CD	FD	C/F
EMR	***	**	*	NS	NS
GLE	***	***	***	**	**
ITC	NS	NS	***	**	NS
PEC	***	***	NS	NS	NS
TSC	**	**	***	***	NS
SCH	***	***	***	**	**

NS = not significant
* = $0.05 \geq p > 0.01$
** = $0.01 \geq p > 0.001$
*** = $p \leq 0.001$

Table 4. Two-way ANOVA test showing the significance of the differences between animals for the tissue morphological parameters.

Muscle	%OFN	%OFA	CD	FD	C/F
EMR	NS	NS	NS	NS	***
GLE	NS	NS	**	***	***
ITC	NS	NS	***	NS	***
PEC	NS	NS	***	***	***
TSC	***	***	***	***	***
SCH	NS	NS	***	***	*

NS = not significant
* = $0.05 \geq p > 0.01$
** = $0.01 \geq p > 0.001$
*** = $p \leq 0.001$

Discussion

Sampling procedure and individual variability

The high levels of significance between most pairs of fields analyzed by the two-way ANOVA test (Table 3) and the results of the Scheffé's multiple comparison tests (Figs. 7 and 8) are a direct consequence of the heterogeneity of the muscles studied. In order to take into consideration the regional variation and the compartimentalization of muscular tissue, we stress the convenience of describing muscle histochemistry throughout the whole muscle section. This is especially necessary for comparative studies which must be carried out by means of a previously designed sampling procedure such as the one used here or those reported elsewhere (Armstrong and Phelps 1984; Bennett and Ho 1988; Laidlaw et al. 1995; Suzuki 1995; Garland et al. 1995). In addition, the significant differences found between animals in CD, FD and C/F (Table 4) suggest a great variability between individuals. For this reason, when low sample sizes are used, it is recommended to prepare balanced and crossed experimental designs considering "animal" factor as block (Torrella et al. 1996).

Fibre types and capillarization in leg muscles

In coot leg muscles, regional differences in capillarization and an uneven distribution of three fibre types (SO, FOG and FG) throughout the cross section are evident (Fig. 4, Table 2). Since the differences in contractile properties found between regions of compartimentalized muscles suggest important functional differences within the same muscle (De Ruiter et al. 1995), our findings should be seen as a reflection of the great variety of activities that coots are able to develop with their legs. Being waterbirds characteristic of large and still waters (Cramp and Simmons 1980), coots must use their legs for aquatic locomotion during different swimming performances. Thus, aerobic zones of internal ITC (I.3, I.4) and anterior GLE (G.1-G.3), with %OFN over 60% and CD ranging from 1,100 to 1,450 cap/mm^2 (Fig. 4, Table 2), are presumably recruited during steady paddling swimming. Coots also regularly dive for short times, no more than 20 s, normally to depths of 1-2 m (Cramp and Simmons 1980; Del Hoyo et al. 1996). Since diving birds metabolise aerobically for dive durations under 15 s (Butler 1991) and coots use only the feet to provide the propulsive force during underwater locomotion (Neu 1931), it is proposed that aerobic parts of ITC and GLE are also used for feeding dives. Muscle regions of external ITC (I.1, I.2) and posterior GLE (G.5-G.7) with clear anaerobic characteristics such as %OFN under 50% and CD generally under 1,000 cap/mm^2 (Fig. 4, Table 2), may be used for bursts of

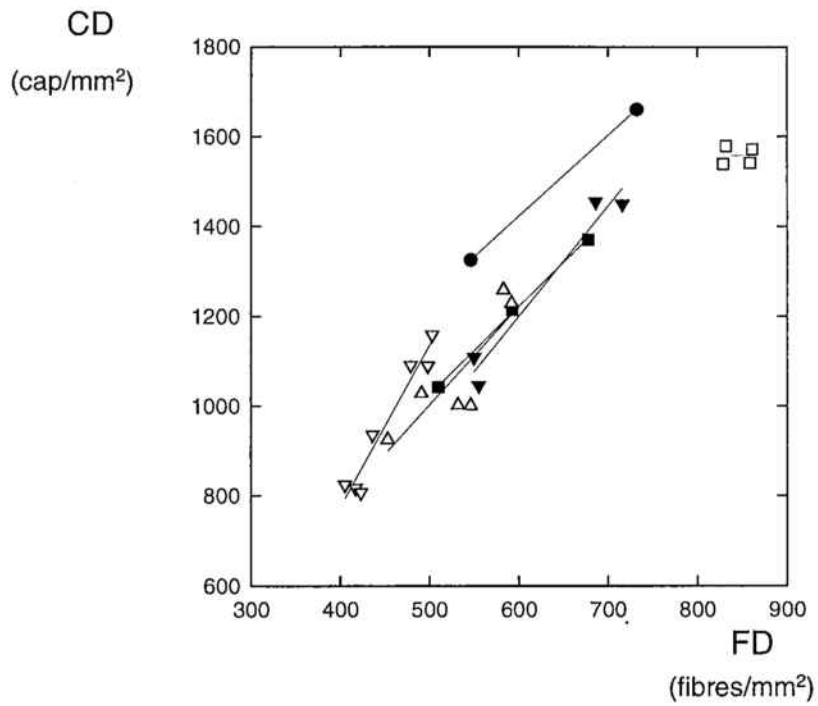

Figure 6. Relationship between fibre density (FD) and capillary density (CD) showing the regression lines for each muscle. Each field is represented by a symbol: GLE: ▽ ; SCH: △ ; PEC: □ ; TSC: ■ ; EMR: ● ; ITC: ▼.

swimming when the paddling stroke frequency increases (Aigeldinger and Fish 1995). The use of burst swimming may be especially relevant during the wing-moulting period when coots remain flightless for up to four weeks owing to the simultaneous lose of the remiges (Cramp and Simmons 1980; Del Hoyo et al. 1996). During this period these birds must rely exclusively on leg locomotion for important activities such as escaping from predators. Great amounts of anaerobic power performed by the legs may also be required during the breeding season when coots behave highly territorially and display charging-attacks involving high speed swimming and violent striking with the feet (Cramp and Simmons 1980; Del Hoyo et al. 1996).

ITC has considerably higher FD than GLE (Table 2). This indicates that ITC has narrower fibres than GLE which may be correlated with the locomotor function of these muscles. The paddling stroke in aquatic birds is divided into two phases which are not symetrical: a power phase for thrust production and a recovery phase which prepares the limb for the next stroke (Clark and Fish 1994). During the power phase the webbed foot (in common coots it is lobed) is fully spread to accelerate water posteriorly, whereas it is folded during the recovery phase to reduce drag forces. Since fibres with large cross-sectional areas have a great capacity for force development (Lucas et al. 1987; Elzinga et al. 1989), its presence in GLE (low FD, Table 2) could be useful in order to overcome high water drag forces, when extending the tarsometatarsus during stroke paddling. However, in the recovery phase, when femur protaction is undertaken by ITC, large fibre cross-sectional areas are not needed (high FD, Table 2) owing to the reduced fluid forces exerted by the folded feet.

In accordance with other reports on avian leg musculature (Maier 1983; Suzuki et al. 1985; Turner and Butler 1988; Suzuki and Tamate 1988; León-Velarde et al. 1993; Torrella et al. 1996), we have found considerable amounts of

M. Triceps scapularis

	3	2	1		1	2	3
	*	NS		1		NS	NS
FD	NS		NS	2	NS		NS
		NS	NS	3	*	NS	

% OFA

C/F CD

M. Pectoralis

	4	3	2	1		1	2	3	4
	NS	NS	NS		1		NS	NS	***
FD	NS	NS		NS	2	NS		NS	***
	NS		NS	NS	3	NS	NS		***
		NS	NS	NS	4	NS	NS	NS	

% OFA

C/F CD

M. Scapulohumeralis

	6	5	4	3	2	1		1	2	3	4	5	6
	NS	NS	NS	NS	NS		1		NS	**	NS	**	NS
	NS	NS	NS	NS		NS	2	NS		***	NS	***	NS
	NS	NS	NS		NS	NS	3	NS	NS		***	NS	***
FD	NS	NS		NS	NS	NS	4	NS	NS	NS		***	NS
	NS		NS	NS	NS	NS	5	NS	NS	NS	NS		***
		*	NS	NS	NS	NS	6	NS	NS	NS	NS	NS	

% OFA

C/F CD

Figure 7. Multiple comparison test by the Scheffé's procedure in fields of muscles involved in aerial locomotion. Vertical and horizontal rows converge in small cells inside of which the results of the comparison test between the two convergent fields are given: NS = non significant; * = $0.05 \geq p > 0.01$; ** = $0.01 \geq p > 0.001$; *** = $p \leq 0.001$.

SO fibres in the leg muscles studied (Fig. 4). Their oxidative character and mATPase behaviour (Table 1) suggest that these fibres are mainly recruited for postural roles and in order to maintain the tensions during leg movements (Suzuki et al. 1985). The finding of the SO fibres situated in muscle regions closer to the bone (Fig. 4) reinforces this statement. There are many situations in coot's life when the role of these fibres may be important, especially in activities performed on land where coots occasionally feed, remain to oil and preen, roost and where soliciting and copulation also occur (Cramp and Simmons 1980; Del Hoyo et al. 1996).

M.Iliotibialis cranialis

	4	3	2	1		1	2	3	4
	NS	*	NS		1		NS	NS	NS
FD	NS	*		NS	2	NS		NS	NS
	NS		NS	NS	3	*	**		NS
		NS	NS	NS	4	*	**	NS	

C/F CD % OFA

M. Gastrocnemius lateralis

	7	6	5	4	3	2	1		1	2	3	4	5	6	7
	NS	NS	NS	NS	NS	NS		1		NS	NS	NS	**	**	*
	NS	NS	NS	NS	NS		NS	2	NS		NS	*	***	***	***
	NS	NS	NS	NS		NS	NS	3	NS	NS		**	***	***	***
FD	NS	NS	NS		NS	NS	NS	4	NS	*	NS		NS	NS	NS
	NS	NS		NS	NS	NS	NS	5	**	***	**	NS		NS	NS
	NS		NS	NS	NS	NS	NS	6	**	***	**	NS	NS		NS
		NS	NS	NS	NS	NS	NS	7	**	***	**	NS	NS	NS	

C/F CD % OFA

Figure 8. Multiple comparison test by the Scheffé's procedure in fields of the leg muscles. Vertical and horizontal rows converge in small cells inside of which the results of the comparison test between the two convergent fields are given: NS = non significant; * = $0.05 \geq p > 0.01$; ** = $0.01 \geq p > 0.001$; *** = $p \leq 0.001$.

Fibre types and capillarization in PEC muscle

The four PEC fields had CD and FD values among the highest of all the muscles studied (Table 2). Moreover, with the exception of P.4 which has a discrete presence of SW fibres, all PEC muscle is homogeneous in fibre-type composition: it has exclusively FOG fibres (Fig. 2) with a very high SDH staining intensity as compared to FOG fibres of other muscles (Fig. 5). These characteristics are frequently found in muscles having aerobic fibre demands and high fibre metabolism such as in PEC of strong flier birds (George and Berger 1966; Lundgren and Kiessling 1988; Suarez et al. 1991; Mathieu-Costello et al. 1996a); in PEC muscle of bats (Foehring and Hermanson 1984; Mathieu-Costello et al. 1992; 1994b); in the red muscle of tuna, a high-speed swimming fish (Mathieu-Costello et al. 1993; 1996b); and in small mammals such as shrews, which have very high metabolic rates (Savolainen and Vornanen 1995). In all these cases high vascularization ensures an elevated oxygen supply to the active muscles (Schimidt-Nielsen and Pennycuik 1961; Romanul 1965); high FD, indicating a fibre-type population with small diameters, give a reduced fibre diffusion distance from capillaries to mitochondria (Banchero 1975; Sullivan and Pittman 1987; Lundgren and Kiessling 1988); and the dark SDH staining of PEC fibres presumably indicates high SDH activities and large mitochondrial volumes, typical of muscles with great capacities for oxygen consumption (Weibel 1984). The capillarization and fibre types that we have found in the coot PEC muscle seem fitted to attain the high energetic demands elicited by the flapping flight, with fast wing movements, characteristic of these birds. In fact, common coots are strong fliers, as can

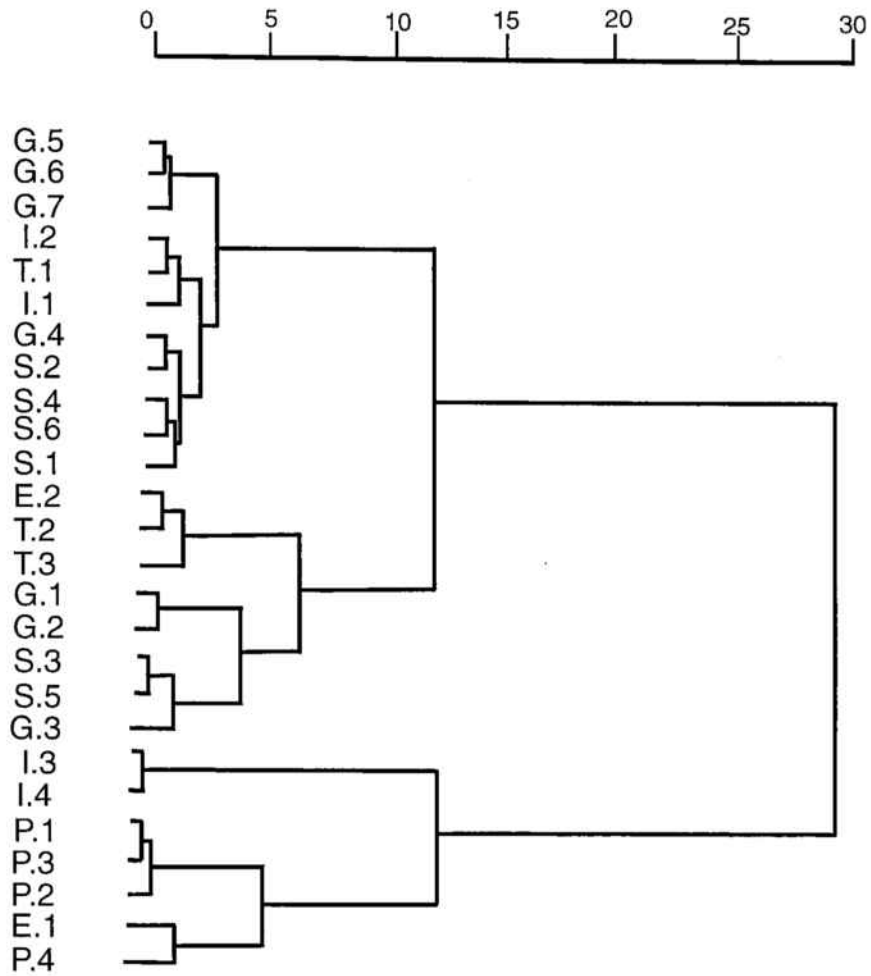

Figure 9. Cluster analysis of all the fields studied using the squared Euclidean distance and the complete method. On the left, fields are named by the first letter of the muscle followed by the number of the field (Figures 2-4).

be demonstrated from the recoverings of ringed birds. Unpublished files from the *Grup Català d'Anellament* (Catalan Bird Ringers Group) contain reports of individuals ringed as far from the Ebre Delta as 1,400 km (near Prague, Czech Republic) and the winter recoveries of coots ringed in localities 700 km away are common. In some instances, these distances are covered in only a few days, as demonstrated by the recovery of a bird ringed in Oberkirch (Switzerland), which was found six days later in the Ebre Delta, 929 km away. Bernis (1966) also quotes distances of 730 km covered in 30-40 hours and 650 km in less than four days for the European common coot population. Moreover, even the coot's native population performs eventual dispersions attaining considerable distances (Bernis 1966). The aerobic SDH profile and the dark Sudan B stainings of FOG fibres of the coot PEC suggest the use of fatty acids as substrates for oxidative fuel provision during these long migratory flights (Berthold 1975; Weber 1992).

The homogeneity in fibre-type distribution found in the PEC muscle (Fig. 2) contrasts with the remarkable degree of heterogeneity of the other muscles studied. In fact, homogeneous muscles are scarcely found in birds and, when present, they play a very especialized role (Nene 1977; Hikida 1981; Meyers 1992a,b; Torrella et al. 1993a). The requirement for high-frequency wing beats and the need for minimizing body mass in a flying animal may produce muscles relying on a "one-geared" system of fibres (Foehring and Hermanson 1984). Moreover, probably as an environmental adaptation for

feeding in shallow waters by short dives, common coots, as diving ducks (Raikow 1973), present small wing areas in relation to its body weight. This could contribute to reducing the bird's buoyancy index and the hydrodynamic drag, although it results in a relatively large wing loading. Such a morphological attribute is adventageous for diving but not for flying since it elicits high energetic costs to perform flapping flight (Rüppell 1977; Gabrielsen et al. 1991). It is likely that all these facts exclude the possibility of having FG fibres in the coot's PEC.

It is widely agreed that FG fibres of bird PEC muscle are used during take-off and landing, when high power output is necessary to beat the wings (Rosser and George 1986a; Welsford et al. 1991). During take-off the absence of FG fibres in coot PEC is supplied by the FG fibres of the leg muscles, the feet and legs acting as an accessory motor and propelling the bird forward until a speed at which the wings can take over is reached. To land on water, coots also solve the inconveniences of lacking FG in PEC by running a short distance over the water surface (Rüppell 1977).

The absence of FG fibres and the presence of FOG of small size has also been reported in the PEC muscles of a common coot's very close relative bird: the Andean coot, *Fulica americana peruviana* (León-Velarde et al. 1993). Moreover, C/F ratio value in the PEC of the Andean coot (1.93) is also very similar to that reported here for the common coot (averaging 1.87, Table 2). However, differences in CD are evident since León-Velarde et al. (1993) found 2,477 ± 367 cap/mm^2 considerably greater than our highest value (1,579 ± 169 cap/mm^2, Table 2). It is improbable that these differences could be attributed to the histochemical methods used in staining capillaries. Our method (Fouces et al. 1993) is based on that published by Rosenblatt et al. (1987) who did not find significant differences in CD between their technique and the amylase-PAS method of Andersen (1975) used by León-Velarde and coworkers. We believe that factors of environmental adaptation could account for the CD difference between both species of coots. As León-Velarde et al. (1993) concluded in their work, the Andean coot shows a genetic adaptation to altitude hypoxia as deduced from low P_{50} values, the lack of polycythemia response to high altitude and the small fibre diameters. Relatively high CD in PEC muscle of Andean coots, even in the individuals born at sea level, could be another genetic factor that may contribute to the toleration of low environmental O_2 pressures.

The deep part of PEC muscle (P.4) presents a low proportion of an unusual slow fibre type, SW (Table 1, Fig. 5), which breaks the uniformity of fibre-type composition in all the coot PEC (Fig. 7, Table 3). The controversy about the twitch or tonic nature of the avian slow fibres is currently an open debate (Barnard et al. 1982; Morgan and Proske 1984; Rosser and George 1986a; Torrella et al. 1993a) and beyond the scope of this work. In any case, slow fibres found in the other muscles of the common coot (SO, Table 1) and in muscles of other bird species (Suzuki 1978; Rosser and George 1985; 1986b; Rosser at al. 1994) have moderate to high oxidative levels. These slow fibres (either twitch or tonic) are present in PEC deep bellies of some soaring birds and a postural function is attributed to them during flight (Rosser and George 1986b; Rosser et al. 1994). The presence of considerable amounts of slow fibres in PEC muscle of flightless birds such as the kiwi, *Apteryx australis mantelli* (McGowan 1982); the emu, *Dromaius novaehollandiae*, (Rosser and George 1984); and the ostrich, *Struthio camelus* (Rosser and George 1985) suggests a functional significance of these fibres in keeping the wings locked (Rosser and George 1984; 1985), especially when they run. To our knwoledge only Rosser et al. (1987) reported in an avian PEC muscle an anaerobic slow type, which was found in a very localized zone of deep PEC muscle of the Japanese quail (*Coturnix japonica*), in a proportion of 7%. It is difficult to attribute a function for the SW fibres in coot's PEC, but we believe they could play a role during feeding dives. Before diving the bird presses the air out of the feathers to reduce buoyancy (Del Hoyo et al. 1996), which could be reinforced by tightening the wings against the body. Since the dives last for only a few seconds these slow fibres presumably do not need an

aerobic metabolism to efficiently maintain the pressure of the wings against the body during diving. A similar function may be held by anaerobic slow fibres in quails which must keep the wings close to the body when running through the vegetation, in order to protect them from damage and to facilitate its advance.

Fibre types and capillarization in wing muscles

Tissue morphological parameters in wing muscles other than PEC reflect a marked oxidative trend. EMR muscle presents in field E.1 the highest CD found in this study (1,660 cap/mm^2, Table 2) with a minimum value of 75% in %OFN (Fig. 2). Moreover, TSC muscle, except for its most posterior field T.1, has also high percentages of FOG fibres (Fig. 3) and great CD (Table 2). SCH, even in its dorsal zones, have %OFN values over 60% (Fig. 3) and CD over 1,000 cap/mm^2 (Table 2). These results contrast with those we obtained for mallard ducks in a similar study (Torrella et al. 1996). In mallard's EMR muscle the maximum %OFN value was around 75% with CD barely exceding 1,000 cap/mm^2. TSC and SCH had a greater proportion of FG fibres and lower CD in all the fields sampled. These differences could be a consequence of differences in wing beat frequency between both species, since common coots have higher wing beat frequencies than mallards (Viscor and Fuster 1987). This feature presumably demands the more developed capillary supply and the presence of greater amounts of FOG fibres able to support the high oxidative needs in oxygen uptake during flapping flight in PEC and wing muscles. Probably as a consequence of the low population of FG fibres in coot's wing musculature, these birds have dificulties in performing non-steady flapping flight (take off, alighting on water and accelerating or decelerating flight). TSC, SCH and EMR are highly active during these phases of flight (Dial 1992) and, although some parts of them (posterior TSC and dorsal SCH) may be involved in these kinds of flight, an extra power from the coot's legs and feet must be required, as has been already discussed above.

SO fibres in the antero-ventral part of the coot's SCH muscle (Fig. 3) are likely to have a postural role in maintaining the wings folded. Moreover, the presence of a slow component in SCH may have a steadying influence on the shoulder joint when PEC muscle moves the brachium during flight (Nene and Naik 1986). Similarly, the SO fibres found in both muscle bellies of coot's EMR (Fig. 2) may function to stabilize the wrist joint during flight (Meyers 1996).

General considerations on fibre types and capillarity

The cluster analysis shows the presence of three groups of fields with gradual oxidative characteristics: anaerobic, aerobic and highly aerobic fields are found distributed throughout all the muscles studied (Fig. 9). This is a result of the differences in fibre-type composition within muscular regions specialized for diverse functional capabilities and is further demonstrated by the finding that, in the cluster analysis, fields containing fast fibres are distinguised from those with slow ones in all the subgroups. Figure 9 matches the plot shown in Figure 6 where gradual relationships between FD and CD within heterogenous muscles are reflected in the similar slopes of the regression lines. Taking all the fields together, it is a general trend that fields with lower FD (larger FCSA) had the lowest CD, whilst those with high FD values (narrower fibres) had the highest CD. This finding demonstrates a linear relationship between CD and FD (or mean FCSA) and reflects that, in all coot muscles studied, a perfect adjustment between fibre diameter and vascularization is achieved in order to obtain the oxygen for mitochondrial supply. This strategy seems a suitable way to cope with the rigid aerobic constraints that flying and diving impose upon coot's physiology.

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VI

Capillarity and fibre types in locomotory muscles from wild yellow-legged gulls (*Larus cachinnans*).
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Capillarity and fibre types in locomotory muscles of wild yellow-legged gulls (*Larus cachinnans*)

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Abstract

Capillarity and fibre-type distributions in six locomotory muscles of wild yellow-legged gulls (*Larus cachinnans*) were analyzed histochemically using a sampling procedure that describes each muscle extensively. Fast fibres were present in all the muscles surveyed, whilst slow fibres lacked in muscles triceps and pectoralis. In contrast to muscles involved in non-flight locomotion, the four flight muscles exhibit a higher percentage of fast oxidative glycolytic fibres (>70%) and a lower percentage of slow oxidative fibres (<16%). Capillary densities ranged from 816 to 1233 capillaries mm^{-2} having the highest value in the pectoralis. In this muscle, the fast oxidative glycolytic fibres had moderate stainings for succinate dehydrogenase and relatively large fibre sizes, as deduced from the low fibre densities. All these findings are seen as an adaptive response for gliding. During this mode of flight the wing is extended by muscles that contract isometrically. This activity, although requiring endurance work (supplied by fast oxidative glycolytic fibres), involves low energy demands that are compatible with the range of capillary and fibre densitites found. Both leg muscles studied included a considerable population of slow oxidative fibres (>14% in many regions) suggesting that they are adapted to postural activities. Regional variations in the relative distributions of fibre types in muscle gastrocnemius may reflect different functional demands placed on this muscle during terrestrial versus aquatic locomotion. The predominance of oxidative fibres with moderate capillary densities is probably a consequence of the buoyant swimming performed by gulls. Their leg muscles may be mainly adapted for slow swimming rather than for other aquatic locomotory capabilities such as endurance or sprint swimming.

Key words Locomoroty muscles · Fibre types · Capillarity · Comparative approach · Yellow-legged gull (*Larus cachinnans*)

Introduction

The varied lifestyles of different vertebrates, and the wide range of locomotory modes by which vertebrates move, is reflected in considerable structural diversity of muscles (Hermanson et al. 1993; Patak and Baldwin 1993; Reed et al. 1994; Savolainen and Vornanen 1995). Although much of the investigative work in muscle physiology has been carried out on mammalian species, some reports have been published relating muscle structure and function in other vertebrate groups. In birds, most studies of locomotion have focused on flight muscles (see for example, Rosser and George 1985, 1986; Rosser et al. 1987; Lundgren and Kiessling 1988; Tobalske 1996). The energetic demands required by flapping and gliding, the two major types of bird flight, are very different (Baudinette and Schmidt-Nielsen 1974; Goldspink et al. 1978; Butler and Woakes 1980; Meyers 1993). These are reflected in the higher physiological needs associated with flapping flight in the fibre-type composition, capillary supply, and architecture of the flight muscles. For example, the occurrence of capillary manifolds in pigeon pectoralis muscle may increase the area for oxygen diffusion

(Potter et al. 1991) and the presence of high capillary densities and low fibre cross-sectional areas in pigeon and hummingbird pectoralis (see Suarez 1992; Mathieu-Costello et al. 1994) may improve oxygen arrival to mitochondria.

In addition to flight, birds also use many other forms of locomotion including walking, running, diving, and different ways of swimming (for review, see Butler 1991). These locomotory behaviours also have an important role in shaping the structural characteristics of avian muscles, especially those of the pelvic appendage. In spite of the importance of these forms of locomotion, only a few papers dealing with muscles other than those involved in flight are available from the literature (Suzuki et al. 1985; Boesiger 1986; Suzuki and Tamate 1988; Patak and Baldwin 1993).

To supplement our knowledge of bird muscle structure, and its ecophysiological implications, this paper presents our findings on fibre types and capillarization of four wing and two leg muscles of a wild species of bird with a typical pattern of gliding flight, the yellow-legged gull (*Larus cachinnans* Pallas).

Material and Methods

Animals

Six yellow-legged gulls (*Larus cachinnans*) of both sexes with a mean body weight of 1,062 ± 63 g (± SEM) were used for this study. Wild animals were obtained from the Medes Islands (Girona, Spain) in April 1993, during a campaign for the control of the population of this species undertaken by wildlife management technicians of the *Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya.*

Muscles

The following six muscles, according to the nomenclature of Vanden Berge (1979), were selected for this study. Four of them are active during flight: muscle pectoralis (PEC), muscle scapulotriceps (TSC), muscle scapulohumeralis caudalis (SCH) and muscle extensor metacarpi radialis (EMR); two are involved in aquatic and terrestrial locomotion: muscle iliotibialis cranialis (ITC) and muscle gastrocnemius lateralis, pars externa (GLE). Figure 1 shows the exact position of each muscle.

PEC arises from the carina and inserts on the deltoid crest of the humerus (George and Berger 1966). This muscle provides the power for the downstroke of the wing during flapping flight by depressing the humerus (Hartman 1961), and the deep part of this muscle supports the body during gliding flight by maintaining the wings outstretched (Meyers 1992, 1993). EMR has two distinct bellies, with their origins from the lateral epicondyle of the humerus. These two bellies are fused near the elbow joint and insert by a common tendon on the extensor process of the carpometacarpus (George and Berger 1966; Hudson et al. 1969). EMR extends and stabilizes the wrist during different phases of flapping flight (Dial 1992). TSC arises from the inferolateral surface of the scapula and inserts by a strong tendon on the proximal end of the coronoid process of the ulna (George and Berger 1966; Hudson et al. 1969). TSC is the principal extensor of the elbow, maintaining wing extension during gliding flight (Meyers 1992, 1993) and extending and stabilizing the elbow during flapping flight (Dial 1992). SCH arises with a fleshy origin from the lateral surface of the scapula and has a fleshy insertion on the bicipital crest of the humerus (George and Berger 1966; Hudson et al. 1969). It is probably involved in retracting and adducting the humerus (Meyers 1992), and in rotating the wing during the final half of the downstroke in flapping flight (Dial 1992). ITC is a straplike muscle which arises from the dorsal crest of the ilium and inserts on the patellar tendon and the anterior tibial crest (Hudson et al. 1969), functioning to protract the femur (Cracraft 1971). GLE has its origin on the surface proximal to the fibular condyle of the femur and inserts on the most lateral part of the Tendo Achilis (George and Berger 1966), having a predominant role in extending the tar-

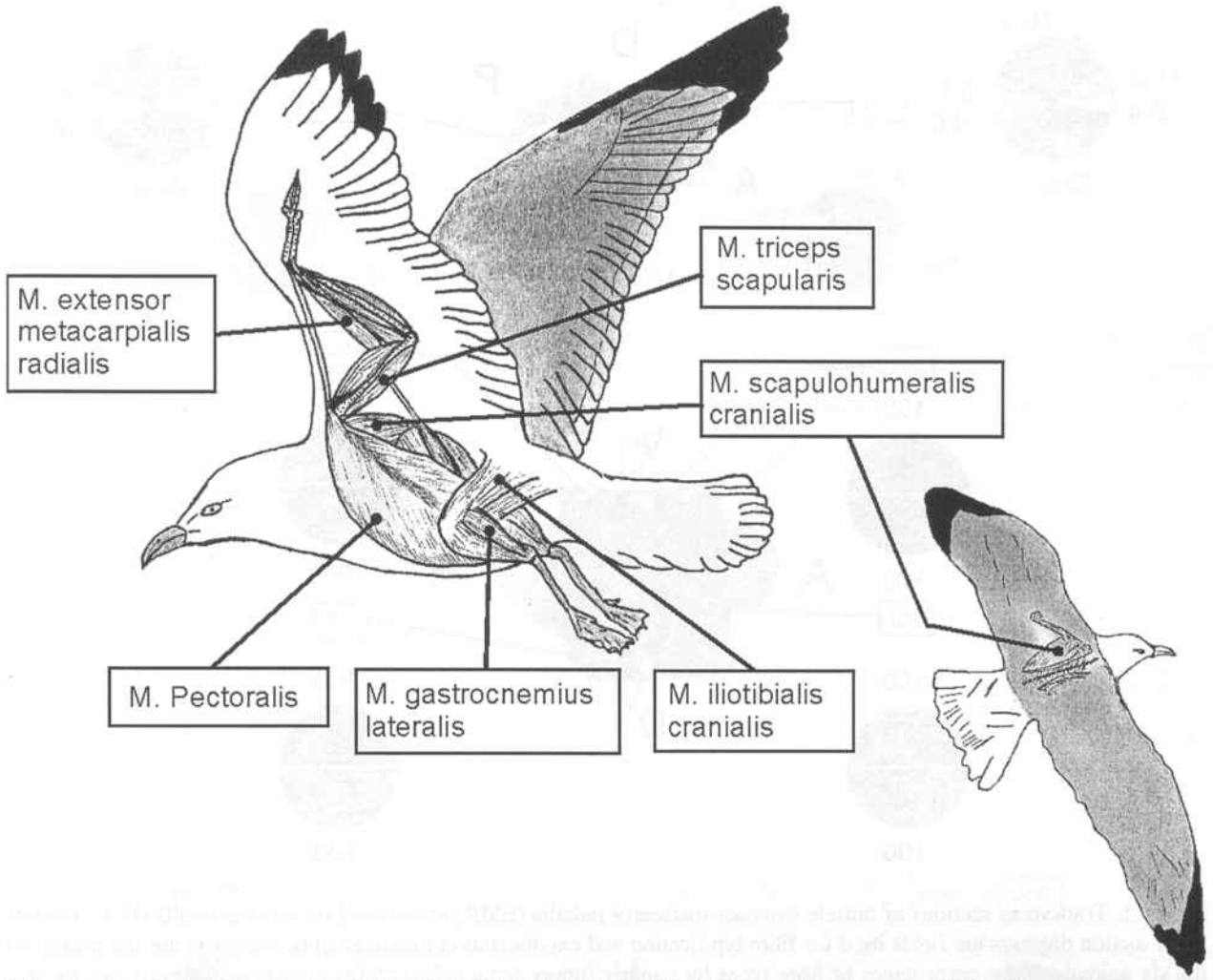

Figure 1. Position of the muscles PEC, EMR, TSC, ITC, SCH and GLE from a lateral view and SCH from a dorsal view.

sometatarsus (Cracraft 1971).

The whole muscles were completely excised from each gull except for the samples from PEC, which were selected from the mid-belly of the muscle, taking special care to dissect them out entirely from the superficial to the deep part. Muscles were marked before excising in order to determine sample orientation when processing.

Histochemical Analysis

After their removal, muscles were frozen in 2-methylbutane cooled to -160°C and stored in liquid nitrogen until sectioning, which was carried out in a cryostat (Reichart, Jung) at -20°C. Transverse serial sections of 14 to 20 µm thick were cut from the muscle equatorial zone, and mounted on 2% gelatinized slides. They were subsequently incubated for 5 min in a buffered fixative (Viscor et al. 1992). There-after, the following histochemical assays were performed: 1. Succinate dehydrogenase, SDH (Nachlas et al. 1957). 2. α-glycerophosphate dehydrogenase, GPDH (Wattenberg and Leong 1960). 3. Myofibrillar adenosine triphosphatase, mATPase (Brooke and Kaiser 1970). 4. ATPase in order to reveal muscle capillaries (Fouces et al. 1993). 5. Sudan Black B (Chiffelle and Putt 1951). 6. Combined mATPase and acetylcholinesterase technique, AChE (Torrella et al. 1993).

Sampling Procedure

Muscle fibre-type heterogeneity is present in most avian muscles (George and Talesara 1961; Suzuki and Tamate 1979). For this

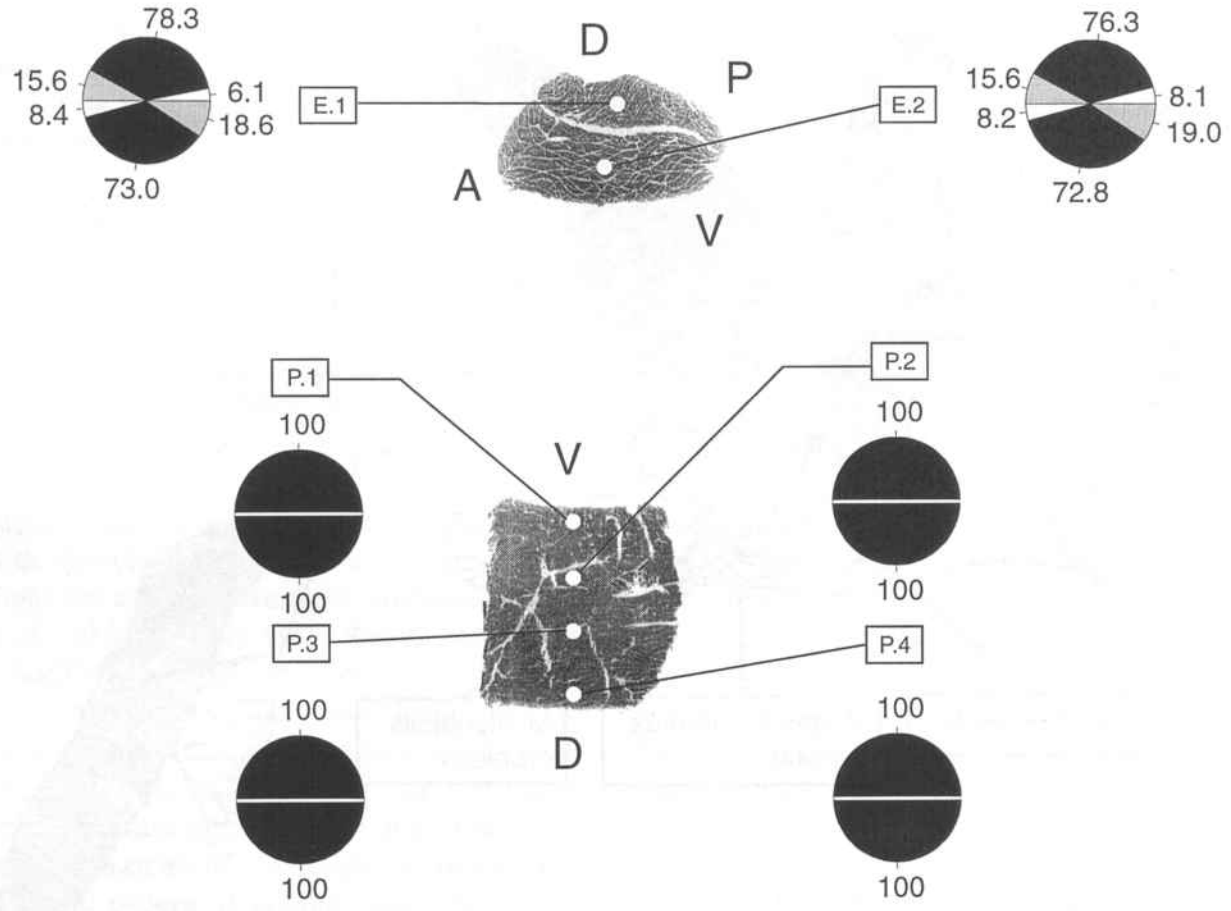

Figure 2. Transverse sections of muscle extensor metacarpi radialis (EMR, above) and muscle pectoralis (PEC, below). Each section displays the fields used for fibre typification and capillarization measurements. Numbers are the means for the six animals of the percentages of fibre types by number (upper semicircles) and percentages of fibre types by area (lower semicircles). Anatomical localization: **D**, dorsal; **V**, ventral; **A**, anterior; **P**, posterior. Sector code colour: FOG, black; FG, grey; SO, white.

reason we designed a sampling protocol for each muscle to allow regional description of fibre-type composition and capillarization in each muscle. Since this protocol has been extensively explained elsewhere (Torrella et al. 1996), only a brief description follows. Transverse sections from each muscle were divided into a two-dimensional grid throughout two-dimensional axes. First we determined the major axis and divided it into several regular intervals. Thereafter, secondary orthogonal axes that transected the divisions were drawn as lines, the total length of which was also divided into two to four regular intervals. This procedure yielded a grid on each sample from which we selected, as measuring zones or fields, some of the areas centered on the intersections (Figs. 2, 3, 4).

As a result of this sampling protocol, 26 muscle fields were used for this study. These muscle zones will be identified throughout the text, tables and figures using the first initial of the muscle name followed by the number of the field (see Figs. 2, 3, 4 for the exact position of each field).

Histology

Fibre types were classified according to the basic scheme of Barnard et al. (1982) for bird skeletal muscle, using the histochemical assays mentioned above as descriptive criteria. We

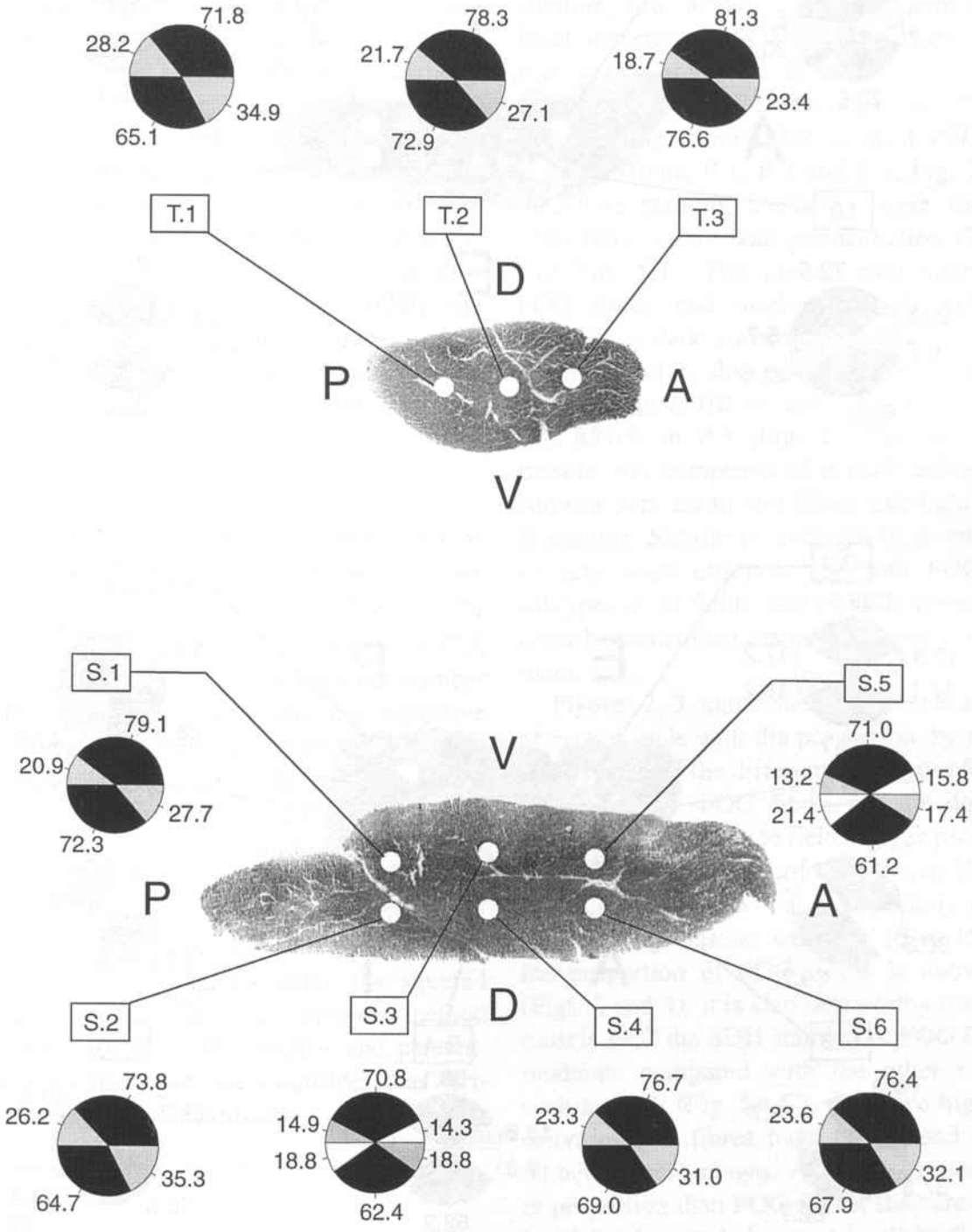

Figure 3. Transverse sections of muscle triceps scapularis (TSC, above) and muscle scapulohumeralis caudalis (SCH, below). Each section displays the fields used for fibre typification and capillarization measurements. Numbers are the means for the six animals of the percentages of fibre types by number (upper semicircles) and percentages of fibre types by area (lower semicircles). Anatomical localization: **D**, dorsal; **V**, ventral; **A**, anterior; **P**, posterior. Sector code colour: FOG, black; FG, grey; SO, white.

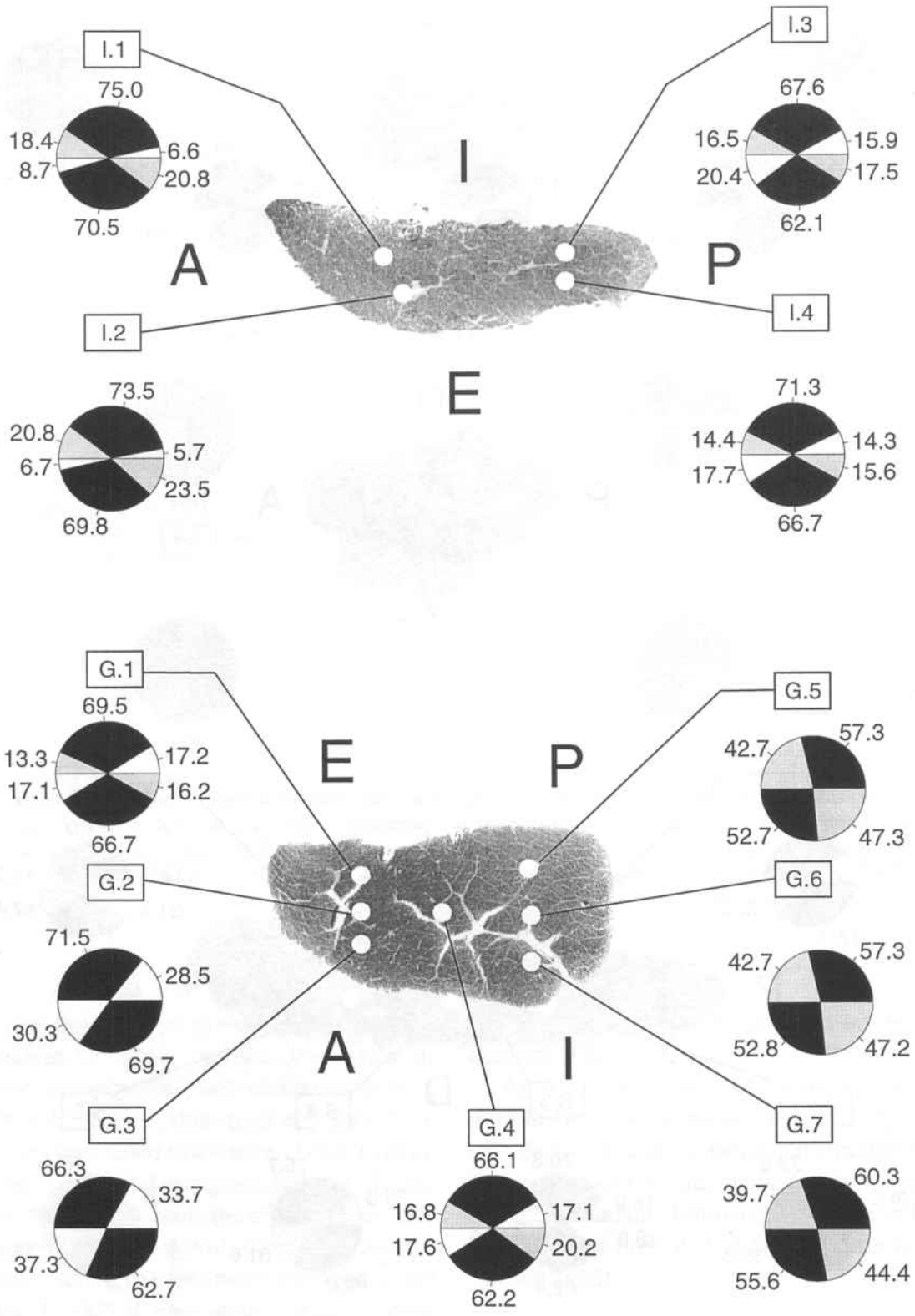

Figure 4. Transverse sections of the muscles iliotibialis cranialis (ITC, above) and muscle gastrocnemius lateralis (GLE, below). Each section displays the fields used for fibre typification and capillarization measurements. Numbers are the means for the six animals of the percentages of fibre types by number (upper semicircles) and percentages of fibre types by area (lower semicircles). Anatomical localization: **I**, internal; **E**, external **A**, anterior; **P**, posterior. Sector code colour: FOG, black; FG, grey; SO, white.

have adopted the fibre-type nomenclature of Peter et al. (1972) because it reflects metabolic and functional characteristics of muscle fibres (see Butler 1991). Measurements were made from photomicrographs taken at a magnification of 80x and 200x using a light microscope (Dialux, Leitz, Wetzlar, Germany) equipped with a camera (Wild, MPS51, Heerburg, Switzerland). Fibre measurements were carried out by means of a digitizer tablet (Calcomp 2318-4, Anaheim, CA, USA) connected to a personal computer using suitable software (Sigma-Scan, Jandel Scientific, Erkrath, Germany). Capillary density (CD), fibre density (FD), capillary-to-fibre ratio (C/F) and fibre type frequencies (both by number and by area proportion) were determined in each field as described by Torrella et al. (1996).

Statistics

Data from all variables are expressed as sample means with 95% confidence limits (means $\pm t_{\alpha,\sigma} \times$ SEM | α=0.05, σ=5, except for ITC and TSC where σ=4). The variables CD, FD, C/F, percentage of oxidative fibre number (%OFN, considering slow and fast oxidative fibres together) and percentage of oxidative fibre area (%OFA) were analyzed using a two-way ANOVA for each muscle taking "field" and "animal" as factors. A multiple comparison test using Scheffé's procedure was performed in order to determine differences in sample means between fields from the same muscle. Finally, a cluster analysis using the squared Euclidean distance and the complete method was developed. CD, FD, %OFA and percentage of slow fibres area were considered as variables after being standardized.

Results

Fibre Types

Table 1 and Figure 5 show the fibre types found in the six muscles studied. In summary, slow oxidative (SO) fibres were characterized by low alkaline and high acidic mATPase stability, moderate to high SDH and low GPDH staining, and multiple innervation. Both fast glycolytic (FG) and fast oxidative glycolytic (FOG) fibres were identified by high alkaline, low acidic mATPase stability, and focal innervation. SDH activity in FG fibres was low as opposed to FOG fibres, where moderate-to-high SDH stainings were found. It is interesting to note that in most PEC areas sampled (fields P.1, P.2 and P.3, Fig. 2), two mATPase staining intensities were found in FOG fibres after alkali preincubation (Table 1 and Fig. 5H). The predominant subtype of FOG fibres had moderate alkali mATPase stability, a dark staining pattern of Sudan B reaction and a distribution throughout PEC muscle from 77.0% in field P.1 to 80.2% in P.2 and 85.6% in P.3 (Fig. 2). The rest of the muscle was composed of a FOG subtype that showed dark alkali mATPase and light Sudan B staining. Similar percentages by number and by area were observed for both FOG fibre subtypes in all fields, and no differences in the other histochemical assays were noted between them.

Figures 2, 3, and 4 show transverse sections of each muscle with the proportion, by number and by area, of the different fibre types for each field sampled. FOG fibres are the dominant fibre type in all muscle fields. Their proportion in number is over 70% of all fibres in 19 of the 26 fields studied, which is especially evident for the wing muscles where in 15 of 15 fields the proportion of FOG fibres is above 70% (Figs. 2 and 3). It is also noteworthy that in the muscle PEC the SDH staining of FOG fibres is moderate compared with the other muscles, such as GLE (Fig. 5A,C), that have high SDH activities. FG fibres have the second highest numerical percentages. Although in much lower proportion than FOG fibres, they are present in all fields sampled except in all PEC muscle (Fig. 2) and in fields G.2 and G.3 (Fig. 4) of the GLE muscle, where they are absent. SO fibres are not found in the PEC and TSC, but are found in all other muscles, generally occupying the parts of the muscle closer to the bone. In ITC proportions of SO fibres were

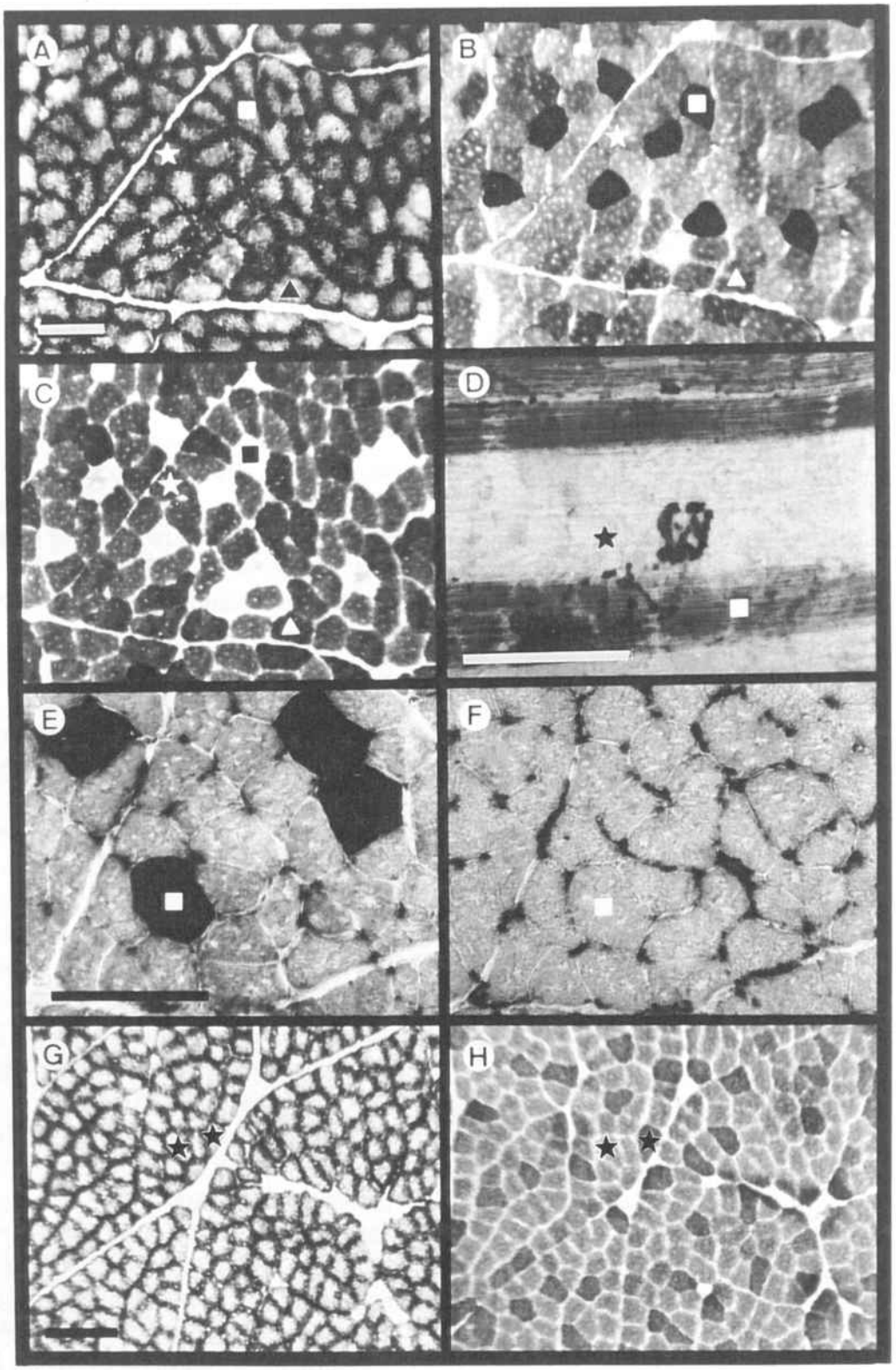

Figure 5. Transverse sections of different gull muscles. (**A, B, C**: GLE; **E, F**: ITC; **G, H**: PEC) processed for mATPase (**C** and **H** preincubated at pH 11.0; **B** and **E** preincubated at pH 4.2), SDH assay (**A** and **G**), and ATPase in order to reveal muscle capillaries (**F**). Micrograph **D** is a longitudinal section of GLE processed for the combined mATPase and AChE method preincubated at pH 4.2. Different fibre types are identified on the micrographs. Fibre type codes: SO, square; FG, triangle; FOG, star. Bar represents 100 μm.

Table 1. Skeletal muscle fibre types based on their histochemical profile and innervation characteristics.

Fibre type	SO	FOG	FG
mATPase			
alkali preincubation	light	dark* (also moderate)	dark
acid preincubation	dark	light	moderate
SDH activity	moderate to high	moderate to high	low
GPDH activity	low	moderate to high	high
Sudan B staining	moderate	dark (also light*)	light
Innervation pattern	multiple	focal	focal
Neuromuscular junction structure	small knobs	"en plaque"	"en plaque"

*Only present in some muscle pectoralis areas (fields P.1, P.2 and P.3, Fig. 2)

about 15% in both posterior fields (I.3 and I.4, Fig. 4), whereas only a 5% was found in the anterior parts of this muscle (I.1 and I.2, Fig. 4). In constrast, GLE had a proportion of 17% to 34% of SO fibres unevenly distributed throughout the anterior and medial zones of the muscle (G.1-G.4, Fig. 4), and lacked SO fibres in the posterior fields (G.5-G.7, Fig. 4). The only wing muscle with a remarkable presence of SO fibres is SCH, which has in its anatomically deepest and most anterior part (fields S.3 and S.5, Fig. 3) a numerical proportion of almost 15%. EMR presented similar amounts of SO fibres, 6% and 8%, in both bellies (Fig. 2).

Tissue Morphological Parameters

Table 2 shows the tissue morphological parameters studied for each field. In all cases where FG fibres are present, the %OFA values are 1% to 9% lower than %OFN, as a consequence of the greater relative size of FG versus oxidative fibres. Table 2 also shows the CD values which had a low range of variation (from 816 to 1,233 capillaries mm^{-2}) among the different muscle regions. Fields from ITC and PEC present higher FD (over 600 fibres mm^{-2}) than the others. This has its reflection in Figure 6A, where these fields are segregated in the upper right part of the plot, and in the cluster, both branching at d=12.9 from the nearest group composed of the fields of TSC and EMR (Fig. 8). A graph including the data obtained in this work on gulls and those from a previous study on mallards (Fig. 6B) shows a greater dispersion of points for mallard muscles because of the greater variations in CD and FD throughout muscles. CD and FD (calculated from C/F original data) in muscle PEC of different bird species are compiled in Table 3.

Table 2. Tissue morphological parameters for each field expressed as sample means with 95% confidence limits. Fields are named by the first letter of the muscle followed by the number of the field (Figs. 2-4).

Field	% OFN	% OFA	CD	FD	C/F
E.1	84.4 ± 10.3	81.4 ± 12.2	1182 ± 57.7	497 ± 81.9	2.42 ± 0.33
E.2	84.4 ± 7.2	81.0 ± 8.5	1156 ± 179.5	520 ± 123.8	2.24 ± 0.62
G.1	86.7 ± 7.5	83.8 ± 8.8	940 ± 78.4	456 ± 61.5	2.08 ± 0.22
G.2	100.0 ± 0.0	100.0 ± 0.0	1066 ± 199.6	534 ± 164.1	2.06 ± 0.33
G.3	100.0 ± 0.0	100.0 ± 0.0	1017 ± 294.2	510 ± 175.0	2.04 ± 0.29
G.4	83.2 ± 7.4	79.8 ± 8.8	945 ± 114.3	522 ± 118.6	1.85 ± 0.29
G.5	57.3 ± 20.4	52.7 ± 22.5	879 ± 188.2	500 ± 122.4	1.78 ± 0.20
G.6	57.3 ± 18.5	52.8 ± 19.3	816 ± 88.1	532 ± 113.4	1.56 ± 0.19
G.7	60.3 ± 17.5	55.6 ± 17.3	865 ± 102.5	510 ± 146.4	1.76 ± 0.33
I.1	81.6 ± 6.1	79.2 ± 6.8	1062 ± 104.1	646 ± 59.3	1.65 ± 0.18
I.2	79.2 ± 8.9	76.5 ± 9.4	1032 ± 100.1	636 ± 77.9	1.63 ± 0.23
I.3	83.5 ± 4.6	82.5 ± 4.8	1094 ± 182.8	626 ± 93.1	1.75 ± 0.11
I.4	85.6 ± 3.3	84.4 ± 3.1	1137 ± 201.9	643 ± 75.0	1.77 ± 0.21
P.1	100.0 ± 0.0	100.0 ± 0.0	1233 ± 113.2	650 ± 94.5	1.91 ± 0.17
P.2	100.0 ± 0.0	100.0 ± 0.0	1210 ± 143.0	665 ± 75.8	1.83 ± 0.21
P.3	100.0 ± 0.0	100.0 ± 0.0	1087 ± 98.4	614 ± 106.3	1.80 ± 0.28
P.4	100.0 ± 0.0	100.0 ± 0.0	1126 ± 131.6	589 ± 105.2	1.95 ± 0.32
T.1	71.8 ± 9.9	65.1 ± 13.7	1119 ± 270.8	554 ± 146.7	2.05 ± 0.47
T.2	78.3 ± 9.0	72.9 ± 12.0	1039 ± 132.8	497 ± 116.1	2.13 ± 0.39
T.3	81.3 ± 6.9	76.6 ± 8.2	1046 ± 201.6	504 ± 130.3	2.11 ± 0.42
S.1	79.1 ± 6.4	72.3 ± 7.9	931 ± 122.4	491 ± 100.9	1.93 ± 0.28
S.2	73.8 ± 7.7	64.7 ± 8.5	929 ± 57.2	489 ± 91.8	1.94 ± 0.28
S.3	85.1 ± 9.3	81.2 ± 11.5	1056 ± 117.0	474 ± 99.8	2.29 ± 0.45
S.4	76.7 ± 8.0	69.0 ± 9.6	924 ± 94.8	519 ± 75.6	1.80 ± 0.22
S.5	86.8 ± 8.8	82.6 ± 11.9	994 ± 97.8	505 ± 71.8	2.00 ± 0.31
S.6	76.4 ± 8.7	67.9 ± 11.3	980 ± 105.2	537 ± 55.4	1.83 ± 0.14

Individual and Regional Muscle Variability

Tables 4 and 5 show the significance of the differences between fields and animals, from a two-way ANOVA test for each parameter and muscle. No results are shown in either table for %OFN or %OFA in PEC because this muscle has exclusively oxidative fibres, and the test is irrelevant. With the exception of GLE and SCH, significant differences between the sampled fields were not found for most parameters when comparing fields from the same muscle (Table 4). Statistically significant differences were found in %OFA and %OFN for TSC and SCH, and in CD and FD for PEC. However, if the influence of the "animal" factor is excluded and a conservative multiple comparison test (such as Scheffé's procedure) is run, no significant differences are evident between fields, an exception being the %OFA in GLE (Fig. 7). FD, C/F and CD were homogeneous enough not to present any significant difference between the seven fields sampled. However, a clear difference for %OFA was evident between the aerobically anterior (fields G.2-G.3, Fig. 4) and anaerobically posterior

Table 3. Muscle cross-sectional capillary and fibre densities of muscle pectoralis of different bird species. The range of CD and FD values is presented when authors did not calculate a mean of the sample.

Bird Species	CD (capillaries · mm^{-2})	FD (fibres · mm^{-2})	Reference
Hummingbird			
Selasphorus rufus (range, n=4)	6,237 - 11,788	3,465 - 7,059[a]	Mathieu-Costello et al. (1992)
Andean coot			
Fulica americana peruviana	2,477	1,283[a]	León-Velarde et al. (1993)
Pigeon *Columba livia*			
Domestic breeds (range, n=12)	1,491-5,680	766 - 2,367[a]	Mathieu-Costello (1991)
Urban	2,362	970	Fouces et al. (1993)
Urban and homing (range)	2,374 - 2,808	1,020 - 1,104	Viscor et al. (1992)
Urban (flying - restricted)	2,075 - 2,429	1,646 - 1,967	Rakušan et al. (1971)
Wild	4,387	2,194[a]	Mathieu-Costello et al. (1994)
Tufted duck *Aythya fuligula*	3,361	1,530[a]	Turner and Butler (1988)
Mallard duck			
Anas platyrhynchos	1,339	930	Torrella et al. (1996)
15 species of passerine (range)	1,400 - 2,500	-	Lundgren and Kiessling (1988)
Black-headed gull			
Larus ridibundus	1,280	695	Viscor et al. (1991)
Yellow-legged gull			
Larus cachinnans	1,164[b]	630[b]	This study

[a] Calculated from CD and C/F

[b] Mean of the four fields sampled (for range values see Table 2)

parts of the muscle (fields G.5-G.7, Fig. 4). Differences with less strict levels of significance ($0.05 \geq P > 0.01$ or $0.01 \geq P > 0.001$) between some fields indicate the presence of a gradient of %OFA throughout the major axis of this muscle. The lack of regional variability in other muscles, demonstrated by these statistical tests (especially for CD, FD and C/F), is also reflected in Figure 6B, where most points relating CD and FD values are concentrated in a relatively small area of the plot. This is especially evident when separated muscles are considered and their respective regression lines, having slopes without any noticeable pattern, are shown (Fig. 6A).

The results of the cluster analysis by the complete method are displayed in Figure 8. Complete linkeage, also called furthest neighbour, often tends to develope a large number of small clusters and a small number of slowly growing major groups. This classification structure matched well with the expected relationship between fields, in accordance with the different muscles and fibre-type compositions. Thus, two groups of fields are segregated at first at an amalgamation distance d=28.3. First, there are those fields from GLE and SCH which presumably have the lowest oxidative capacities because of their lowest CD (816-980 capillaries mm^{-2}). Second, a branch at d=20.9 segregates the remaining fields from GLE and SCH, all of them having considerable amounts of SO fibres, from all the other muscles with higher CD (1,017-1,233 capillaries mm^{-2}). Finally, at d=12.9 fields with high CD and low FD (EMR and TSC) are separated from those with high CD and high FD (PEC and ITC). Similarities between the results of the cluster analysis and Figure 6A are evident. The fields with low FD and low CD (SCH and

GLE) are grouped in the lower part of the plot, occupied by the hollow triangles. A segregation between the fields with higher CD and low FD (EMR and TSC), and those with high CD and high FD (PEC and ITC) is also distinguished in the left and right upper parts of the graph.

Table 4. Two-way ANOVA test showing the significance of the differences between fields for the tissue morphological parameters.

Muscle	%OFN	%OFA	CD	FD	C/F
EMR	NS	NS	NS	NS	NS
GLE	**	***	**	NS	***
ITC	NS	NS	NS	NS	NS
PEC	-	-	**	*	NS
TSC	***	**	NS	NS	NS
SCH	**	***	*	NS	**

NS = not significant
* = $0.05 \geq P > 0.01$
** = $0.01 \geq P > 0.001$
*** = $P \leq 0.001$

Table 5. Two-way ANOVA test showing the significance of the differences between animals for the tissue morphological parameters.

Muscle	%OFN	%OFA	CD	FD	C/F
EMR	NS	NS	NS	*	NS
GLE	**	**	***	***	***
ITC	NS	NS	**	*	*
PEC	-	-	***	***	**
TSC	***	***	**	***	**
SCH	**	**	**	***	***

NS = not significant
* = $0.05 \geq P > 0.01$
** = $0.01 \geq P > 0.001$
*** = $P \leq 0.001$

Discussion

Variability within Muscular Regions and Between Animals

Apart from GLE, all the muscles of the yellow-legged gull studied here have low regional variation throughout their transverse cross-sections (Table 4, Fig. 7). This is in contrast to our findings in mallard duck muscles where, after an identical experimental design, we reported strongly significant differences ($P \leq 0.001$) for almost all pairs of muscle fields. In mallards, we found marked differences for FD, CD and %OFA between posterior and anterior TSC, a pronounced gradient in CD and %OFA from ventral to dorsal parts of the SCH, and statistical significant differences in measures between the anterior and exterior zones of the leg muscles (Torrella et al. 1996). The wide range of CD and FD shown in the relationship between these two parameters for mallard muscles (Fig. 6B) reinforces these statistically verified findings. We think that the locomotory habits of both species discussed below could account for these contrasts.

A great individual variability among wild yellow-legged gulls is present in all the parameters studied (Table 5). This finding agrees with, and reinforces, our previous statement that for low sample sizes the study of morphological muscular parameters must be designed with balanced and crossed experiments that consider "animal" factor as block (Torrella et al. 1996).

Fibre Types and Environmental Implications

Leg muscles

The presence of SO fibres in leg muscles of birds is well documented and it is widely accepted that the SO fibres of leg muscles are recruited mainly for postural activities (Suzuki and Tamate 1979; Suzuki et al. 1982; Li et al. 1988; Butler 1991), although in some cases there is evidence that these fibres may also be active in locomotion (Walmsey et al. 1978). SO fibres range from 10% to 33% in the

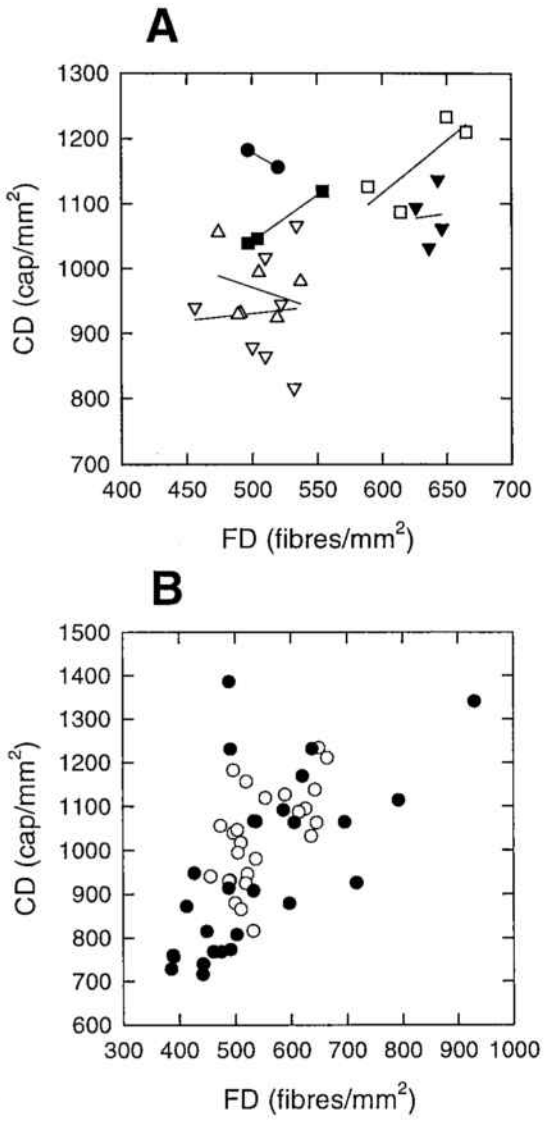

Figure 6. **A** (upper panel). Relationship between fibre density (FD) and capillary density (CD) showing the regression lines for each muscle. Each field is represented by a symbol: GLE: ▽; SCH: △; PEC: □; TSC: ■; EMR: ●; and ITC: ▼. **B** (lower panel). Relationship between FD and CD for the six muscles studied in yellow-legged gulls (○) and mallard ducks (●). Each point represents one field. Note the greater dispersion for the fields of mallard muscles. Data of ducks are from Torrella et al. (1996).

pigeon GLE (Maier 1983; Li et al. 1988; Viscor et al. 1992) and from 16% to 20% in the chicken ITC (Suzuki et al. 1985). Interestingly, higher percentages of SO fibres (more than 50%) are found in leg muscles of passeriformes that have considerable postural requirements during perching (Boesiger 1986). In constrast, SO fibres have not been found in GLE of the emu *Dromaius novaehollandiae*, perhaps reflecting a specialization of the GLE of this bird for function in cursorial activity rather than for postural roles (Patak and Baldwin 1993). An intermediate situation is found in the GLE muscle of ducks, with percentages of SO fibres of 15% in the tufted duck *Aythya fuligula* (Turner and Butler 1988) and 20% in the mallard *Anas platyrhynchos* (Torrella et al. 1996). Although these ducks spend a great deal of time in water using their legs for swimming, some SO fibres must be needed to perform activities on land. Since yellow-legged gulls spend much time loafing and standing (Cramp and Simmons 1985), the presence of high amounts of SO fibres in this species is not surprising. In the present study, SO fibres were found to comprise 20% to 40% of some muscle fields (Fig. 4), suggesting that gull leg musculature is well adapted to postural activities. It is interesting to note that in some regions of gull leg muscles FOG together with SO fibres represent 100% in OFN (Table 2). This exclusive aerobic fibre-type presence contrasts with that found in leg muscles in the ducks which possess a large number of FG fibres (Turner and Butler 1988; Torrella et al. 1996). The duck's ability to perform a wide range of swimming activities (slow, sustained and "sprint" swimming; Aigeldinger and Fish 1995) as opposed to the buoyant swimming of gulls (Cramp and Simmons 1985) may account for these differences. Characteristics of terrestrial locomotion of both species may explain some of the differences in leg muscle structure. Very different requirements of leg musculature between mallards and gulls are evident in two examples. First, mallards nest on the ground, among vegetation or in natural cavities (Del Hoyo et al. 1992), having to reach or leave the nest by land. Conversely, gulls do not use their legs to reach or leave the nest, since it is placed in open spaces, commonly rocky coasts with cliffs (Cramp and Simmons 1985), which are accessible only by air. Second, mallard ducks moult their feathers simultaneously, becoming

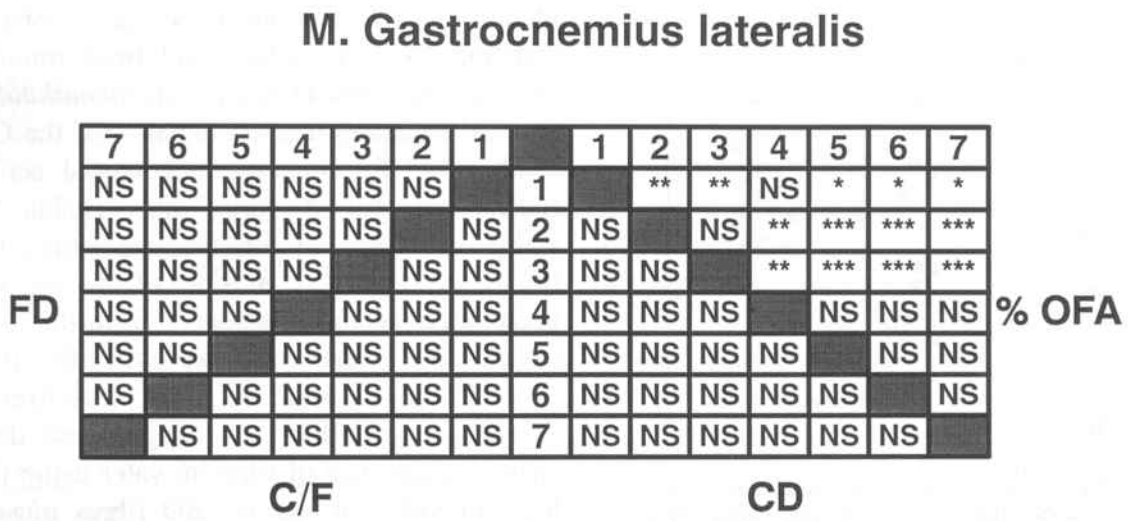

Figure 7. Multiple comparison test by Scheffé's procedure in fields of GLE muscle. Vertical and horizontal rows converge in small cells inside of which the results of the comparison test between the two fields are given: NS = not significant; * = $0.05 \geq P > 0.01$; ** = $0.01 \geq P > 0.001$; *** = $P \leq 0.001$.

flightless for several weeks (Del Hoyo et al. 1992), whereas gulls moult gradually throughout aproximately six months (Cramp and Simmons 1985). During moulting, mallards must rely only on their hindlimbs for locomotion, presumably needing leg muscles with regional variations that enable them to perform a wide range of swimming, walking and running activities.

Wing muscles

Most wing-muscle fields sampled in the present study were devoid of SO. This is in accordance with the results reported by Maier (1983), who found no SO fibres in most forearm muscles of the pigeon. The distribution of SO fibres in the present study suggests that, of the wing muscles, the deep SCH fields (S.3 and S.5, Fig. 3) are the most adapted to a postural role. As we argued for analogous zones of SCH of mallards, where similar quantities of SO fibres were found, this muscle may assit in wing folding by pulling the humerus to the body (Torrella et al. 1996). SO fibres present in numerical percentages of less than 10% in EMR (Fig. 2) may play some role in maintaining extension of the wing and stabilizing it during gliding flight.

In the yellow-legged gull, PEC is comprised solely of FOG fibres, a finding in agreement with previous studies on PEC of other gull species (Rosser and George 1986; Viscor et al. 1991; Caldow and Furness 1993). This contrasts with the PEC muscle of the pigeon or the duck, where two markedly different fibre-type populations (FG and FOG) have been found (for reviews see George and Berger 1966 and Butler 1991). These two populations are proposed to support different functional roles. FOG fibres are thought to be mainly involved in sustained activities during flight whereas FG are thought to be preferentially active during take-off, landing, or sudden changes in direction (Rosser and George 1986; Welsford et al. 1991). Within the gull PEC, however, two populations of FOG fibres differing in alkali stability and lipid densities were observed. This finding is similar to that reported by Caldow and Furness (1993) in the herring gull (*Larus argentatus*). Since differences in shortening velocity between fast fibre types have

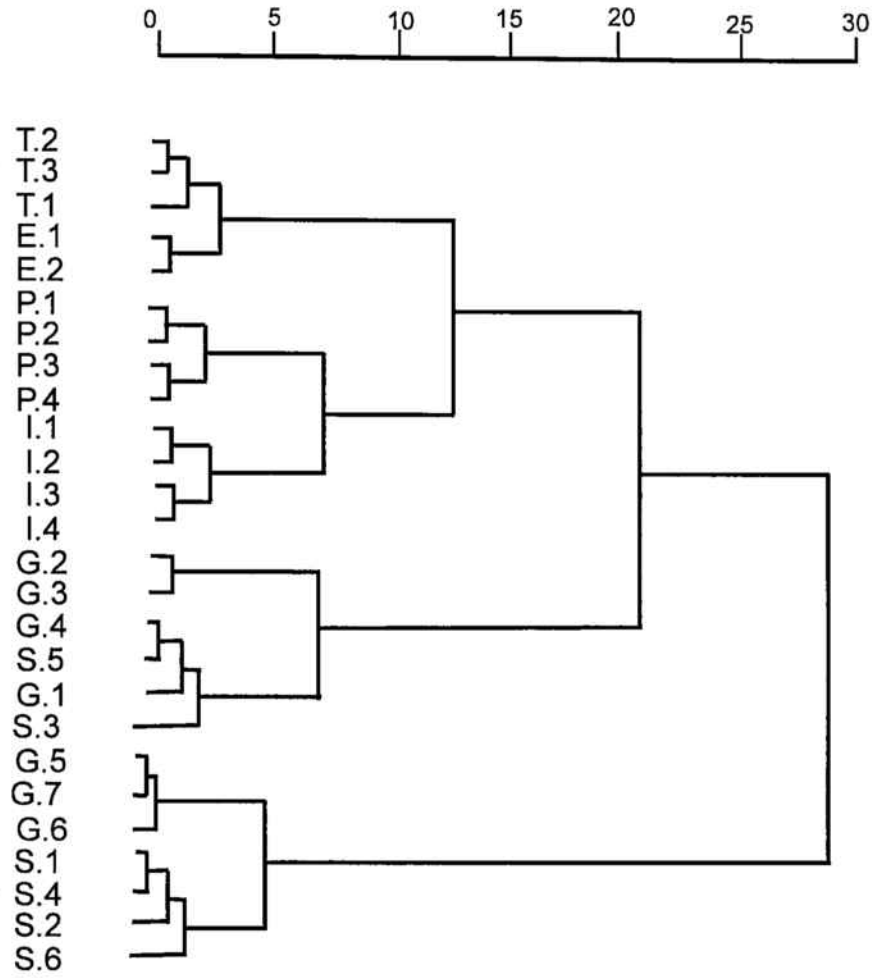

Figure 8. Cluster analysis of all the fields studied by using the squared Euclidean distance and the complete method. On the left, fields are named by the first letter of the muscle followed by the number of the field (Figs. 2-4).

been demonstrated in other muscles (see Pette and Staron 1990; Schiaffino and Reggiani 1994), in the gull FOG fibres of dark ATPase and light Sudan B staining might be recruited during burst activity whereas the other, lipid rich, FOG fibre population, may be preferentially recruited during long glides. Yellow-legged gulls have a low capacity for manoeuvrability in tight spaces (Cramp and Simmons 1985), performing minimal phases of burst activity, and they glide even when preparing to alight on the water (Pennycuick 1987). The differences in the use of different modes of locomotion between birds such as pigeons or ducks and gulls are reflected in the PEC histochemical organization. Flapping flight represents a five- to eight-fold increase in oxygen consumption from resting levels (Tucker 1972), and this high energy consumption is reflected in the high levels of SDH activity found in other flapping-flight birds (Suarez 1992; León-Velarde et al. 1993). Gliding, in contrast, requires only a two-fold increase in oxygen consumption from resting levels (Baudinette and Schmidt-Nielsen 1974) and involves, in the gull, less activity in the PEC muscle than does flapping flight (Goldspink et al. 1978). The lower energy demands of this flight mode may be reflected in the moderate levels of SDH staining found in the PEC (Fig. 5G). This suggestion derives some support from studies of several species of woodpeckers where FOG

fibres with moderate SDH stainings have also been reported (Tobalske 1996). These birds perform intermittent flight -periods of flapping alternated with periods of gliding- which also requires less energy usage than flapping flight (Ward-Smith 1984; Rayner 1985).

In addition to PEC, other wing and shoulder muscles (TSC, EMR and SCH) stand out for their high proportion of FOG fibres: all the 11 fields studied have percentages higher than 70%. This differs from our findings in wing muscles of wild mallard ducks, where a more heterogeneous distribution of FG and FOG fibre types was reported (Torrella et al. 1996). In fact, in most fields the opposite was true, with a more than two fold presence of FG fibres in percentages by area. As discussed for the PEC, these differences may be a consequence of the different flying habits of mallards and gulls. We have proposed that the anaerobic zones found in mallard wing and shoulder muscles could be recruited during non-steady flapping flight (take-off, landing, ascending and descending) when highly energetic bursts are needed (Torrella et al. 1996). The small muscle cross-sectional area occupied by FG fibres in wing and shoulder muscles of yellow-legged gulls may reflect the only occasional need for non-steady flapping flight in this species. Likewise, the presence of a large proportion of FOG fibres may reflect a need for endurance fibres during the enormous length of time that these birds remain in flight and the long-distance displacements that have been reported. The longest distance registered in one day is 300 km (Carrera et al. 1993), and it is common for gulls to fly 160 km from the coast into the continent by following rivers (Carrera et al. 1981). Birds ringed in the Medes Islands colony have been recovered north west in the French Atlantic Ocean between the Basque Country and the Loire Delta, as far as 750 km from birth locality, and south in the Ebre Delta (Carrera et al. 1981, 1993; Le Mao and Yesou 1993).

Capillarization and Behavioural Assotiations

CD in the PEC muscle of yellow-legged gulls is the lowest reported for the PEC muscle of all the bird species listed in Table 3. Interestingly, this table shows that species performing hovering or flapping flight, such as hummingbirds or pigeons, have higher CD in their PEC muscles than the two species of gulls, with a marked gliding flight pattern. Moreover, FD values found in both gull species are the lowest reported in the literature (Table 3). Since high CD and small fibre sizes are known to be related to the high oxidative capacity of muscles (Schmidt-Nielsen and Pennycuick 1961; Romanul 1965), these data may reflect the low oxygen demands imposed in the PEC by gliding flight. Moreover, even in muscle fields having oxidative proportions greater than 70%, lower CD values were found in the other wing muscles (924-1182 capillaries mm^{-2}) as compared to the values shown in Table 3. This could be a consequence of the involvement of wing muscles in maintaining the humerus and the wrist extended during gliding, pulling the tendons by means of isometric contraction, which is less costly than isotonic contraction (Goldspink 1981). These figures agree with some cardiovascular adaptations reported in relation to flight activity in birds. Viscor et al. (1985) found that gulls have lower blood volume per unit of body weight, hematocrit and hemoglobin concentration than pigeons. Butler and Woakes (1980) showed that the heart rate during soaring was 50% less than during flapping flight, indicating a significant reduction in oxygen uptake.

Since fibres of large cross-sectional area produce more force than fibres of small cross-sectional area (Callister et al. 1992), the presence of low FD (large fibre sizes, Table 2) in TSC and EMR and the segregation of both muscles from the others at d=12.9 in the cluster analysis (Figure 8), may be indicative of a functional adaptation of these muscles. None of the wing muscles have great regional variations in capillarization or in %OFA as is deduced from the lack of significant differences found by means of the multiple

comparison test (Scheffé procedure) for all the tissue morphological parameters, and also from the low dispersion of points in the graph that plots CD vs. FD (Fig. 6B). This could be a consequence of the wing muscles' specialization for gliding as a result of an adaptive constraint imposed by the need to be airborne for long periods of time.

Capillarization in ITC muscle presents low regional variations, thus following the pattern described above for its fibre type distribution and, once more, CD does not match the great composition of oxidative fibres (%OFN over 80%, Table 2). This muscle, together with PEC, has the highest FD values, as is seen in Table 2 and Figures 6A and 8, where all their fields are included in the same cluster group at d=12.9. Although they do not have a great CD (1,000-1,200 capillaries mm^{-2}), both muscles presumably adjust their oxygen supply to the fibres by reducing the fibre cross-sectional area, in order to sustain their endurance activities during flying and swimming.

Two reasons may explain the low range of variation in the CD values (816-1,066 capillaries mm^{-2}) within the GLE muscle (Table 2). First, the presence of high percentages of SO fibres in oxidative zones of both gull leg muscles (Fig. 4). Slow fibres have lower energy turnover than fast fibres since they hydrolize ATP at a slower rate (Goldspink 1981). This may result in reduced oxygen and nutrient supply by capillaries to the working muscles, a task that could be affordable by a moderate CD. Second, as mentioned above, the recruitment of fast fibres during the buoyant swimming performed by gulls, although it must require endurance activity, may not be as high-energy demanding as the sustained or short burst swimming activities described in other bird species such as ducks (Aigeldinger and Fish 1995). In fact, ducks have more compartimentalized leg muscles, with higher CD in the aerobic GLE zones and lower CD in the anaerobic parts (Torrella et al. 1996; Turner and Butler 1988), which might cope with a wide range of locomotory activities. However, in gulls, a certain degree of regional variation is present between the anterior and posterior parts of the GLE for %OFA (Fig. 7). This finding is supported by a separation of the posterior GLE from the group of fields, including the anterior GLE at d=28.3 in the cluster analysis (Fig. 8). This slight regionalization indicates that GLE is not as specialized as wing muscles in their function, probably due to their need for both aquatic and terrestrial locomotion.

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VII

Comparative skeletal muscle fibre morphometry among wild birds with different locomotory habits.
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Comparative skeletal muscle fibre morphometry among wild birds with different locomotory habits

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Abstract

Six muscles of the mallard duck (*Anas platyrhynchos*), the common coot (*Fulica atra*) and the yellow-legged gull (*Larus cachinnans*) were morphometrically analyzed, with special emphasis on their functional implications and physiological needs. Oxidative fibres always had significanly lower size than anaerobic fibres, although no differences in the number of capillaries per fibre were found. This resulted in greater capillary counts per unit of fibre area and perimeter in oxidative than anaerobic fibres, which indicates that the incremented demand for oxygen supply may be satisfied by decreasing the size of the muscle fibre rather than by increasing the number of capillaries around it. Greater sizes were found in the oxidative fibres of the pectoralis and triceps of the gull, which correlates with the differences in energetic demands between flapping and gliding flight. Greater fibre cross-sectional areas and perimeters seem suited to afford the long-lasting activity with low metabolic demands required during gliding. By constrast, mallards and coots attain a high oxidative metabolism, during sustained flapping flight, by reducing its size at the expense of a diminished ability for force generation. Between-species comparisons of the hindlimb muscles only yielded differences for the anaerobic fibres of the gastrocnemius, as an important adaptative response to force generation during burst locomotion. Within-species comparison of the leg muscles demonstrated a greater size of the oxidative fibres of the gastrocnemius than those of the iliotibialis muscle, as a consequence of the different mechanical and functional roles of both muscles during swimming.

Key words Mallard · Common coot · Yellow-legged gull · Avian anatomy · Histochemistry

Introduction

Ranvier's pioneering works (1874) on muscle physiology differentiated between two morphological and functional types of muscles: red and white, with slow and fast contractions respectively. After many years of scientific investigation and by means of several biochemical, histochemical and physiological methodologies (Brooke & Kaiser, 1970; Peter et al. 1972; Dubowitz & Brooke, 1973; Burke et al. 1973), anatomists and physiologists have had the opportunity to describe in more detail the differences reported by Ranvier. This has led to the study of muscular properties in relation to muscle fibre composition, capillary supply and other morphometrical parameters such as fibre cross-sectional area and capillary diffusion distances (Plyley & Groom, 1975; Brodal et al. 1977; Gray & Renkin, 1978; Ripoll et al. 1979; Snyder, 1990). Muscles with a great proportion of oxidative fibre types have high oxygen demands and tend to present a high capillary supply, whereas those which rely on glycolytic metabolism consist of anaerobic fibres supplied by poor capillary networks (see for review Hudlická, 1985). It should be noted, however, that many problems appear when detailed studies on muscle tissue are developed. It has been found that fibre types follow a continuum staining pattern when histochemical assays are performed (Dahl & Roald, 1991; Staron & Pette, 1993) which makes it difficult, in some instances, to separate fibres into different types. In addition, nomenclature problems are also evident and, depending on the vertebrate group

studied, various fibre type classifications have been given, which are not always comparable between each other (Barnard et al. 1982; Johnston, 1985; Pette, 1985; Callister et al. 1989).

The fibres of most skeletal muscles not only differ with respect to their contractile function and metabolism, but also to their size and the arrangement and abundance of their capillary supply (Barnard et al. 1971; Burke et al. 1973). The study of the capillary network supplying each muscle has some drawbacks because oxygen supply to muscle cells, which is reached from capillaries by convection and diffusion transport (Groebe, 1992), is a highly complex process. Although many variables have been used in order to quantify the capillary supply in muscle tranverse sections (e.g. capillaries per unit area, capillaries per fibre, number of capillaries around each fibre, intercapillary distances), no single variable adequately describes the physical dimensions and the functional capacity of the capillary network (see Egginton & Ross, 1992). For this reason it is recommended to consider the number of capillaries around each fibre together with other morphometrical fibre parameters, such as fibre areas and perimeters and their relative capillary counts, in order to describe the capillary supply of the individual muscle fibres (Andersen & Henriksson, 1977).

This paper presents the findings of an extensive morphometrical study on muscle fibres of three species of wild birds: the mallard duck (*Anas platyrhynchos*), the yellow-legged gull (*Larus cachinnans*) and the common coot (*Fulica atra*). Six locomotory muscles were used emphasizing their ecophysiological needs after a comparative approach at both the species and fibre type levels.

Materials and methods

Animals and muscles

Adult animals of either sex of three species of wild birds were used for this study (mean ± SEM, body weight): seven mallard ducks *Anas platyrhynchos* (1,009 ± 82 g), six yellow-legged gulls *Larus cachinnans* (1,062 ± 63 g) and six common coots *Fulica atra* (794 ± 53 g). The mallards and the coots were obtained from *Parc Natural del Delta de l'Ebre* (Tarragona, Spain) during January and February of 1992 and 1993. All of them were captured by hunters, supervised by Park guards, under the provision of a scientific collector's permit (no. 5605/92) from the *Direcció General d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya.* The yellow-legged gulls were caught on the Medes Islands (Girona, Spain) in April 1993 during a campaign for the control of the population of this species, undertaken by wildlife management technicians of the *Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya.*

The following six muscles, according to the nomenclature of Vanden Berge (1979), were selected for this study. Four of them are involved in flight: muscle pectoralis (PEC), muscle scapulotriceps or triceps scapularis (TSC), muscle scapulohumeralis caudalis (SCH) and muscle extensor metacarpi radialis (EMR); and two concerned with aquatic and terrestrial locomotion: muscle iliotibialis cranialis (ITC) and muscle gastrocnemius lateralis, pars externa (GLE). Table 1 shows the origin, insertion and function of each muscle according to the current literature.

The whole muscles were excised from each animal in the case of ITC, GLE, TSC, EMR and SCH. Samples from PEC were selected from the mid belly of the muscle, taking special care in dissecting out the muscles entirely from the superficial to the deep part.

Table 1. Origins, insertions and main functions of the six muscles studied in the three bird species.

Muscle	Origin	Insertion	Main function
PEC	Ventrolateral surface of the carina[1,2]	Deltoid crest of the humerus[1,2]	Depressing the humerus in downstroke[3]. Maintaining the wings outstretched in gliding [4]
SCH	Ventrolateral surface of the scapula[1,2,5]	Ventral edge of the humerus[1,2,5]	Retracting, adducting and elevating the humerus. Rotating the wing in downstroke[4,6]
EMR	Dorsal surface of the humerus[1,2,5]	Extensor process of the carpometacarpus [1,2,5]	Extending and stabilizing the wrist during flapping flight [6]
TSC	Cranial ventrolateral tip of the scapula[1,2,5]	Dorsoproximal tip of the ulna[1,2,5]	Extending and stabilizing the elbow during flapping flight and gliding[4,6]
ITC	Anterodorsal iliac crest[1,5]	Patellar ligament anterior tibial crest[1,5]	Protracting the femur[7]
GLE	Fibular condyle of the femur[1]	Lateral part of the Tendo Achilis[1]	Extending the tarsometatarsus[7]

[1] George and Berger (1966), [2] Rosser (1980), [3] Hartman (1961), [4] Meyers (1992), [5] Hudson et al. (1969), [6] Dial (1992), [7]Cracraft (1971).

Histology and sampling procedure

Muscles were marked before excising in order to determine sample orientation when processing. After their removal, muscles were frozen in 2-methylbutane cooled to -160°C and stored in liquid nitrogen until sectioning. Muscles were cut in a cryostat (Reichart, Jung) at -20°C, in transverse serial adjacent sections from their equatorial zone. Sections from 14 to 20 μm thick were mounted on 2% gelatinized slides and incubated for 5 minutes in a buffered fixative (Viscor et al. 1992). Thereafter, the following histochemical assays were developed: 1. Succinate dehydrogenase, SDH (Nachlas et al. 1957). 2. α-glycerophosphate dehydrogenase, GPDH (Wattenberg & Leong, 1960). 3. Myofibrillar adenosine triphosphatase, mATPase (Brooke & Kaiser, 1970). 4. ATPase in order to reveal muscle capillaries (Fouces et al. 1993). 5. Sudan Black B (Chiffelle & Putt, 1951). 6. Combined mATPase and acetylcholinesterase technique, AChE (Torrella et al. 1993).

A sampling protocol for each muscle, in order to describe the morphometrical characteristics of the whole section, was designed (see Torrella et al. 1996). In all cases the transverse sections from each muscle were divided into a grid-like structure throughout two-dimensional axes. First we determined the major axis and divided it into several regular intervals. Thereafter, secondary orthogonal axes that transected the divisions were drawn as lines, the total length of which was also divided into regular intervals. This procedure yielded a grid on each muscle from which we selected, as measuring zones or fields, some of the areas centered on the intersections (see Fig. 1 for the exact position of each field). This sampling protocol resulted in 26 fields which will be designated throughout the text, tables and figures using the first initial of the muscle name followed by the number of the field.

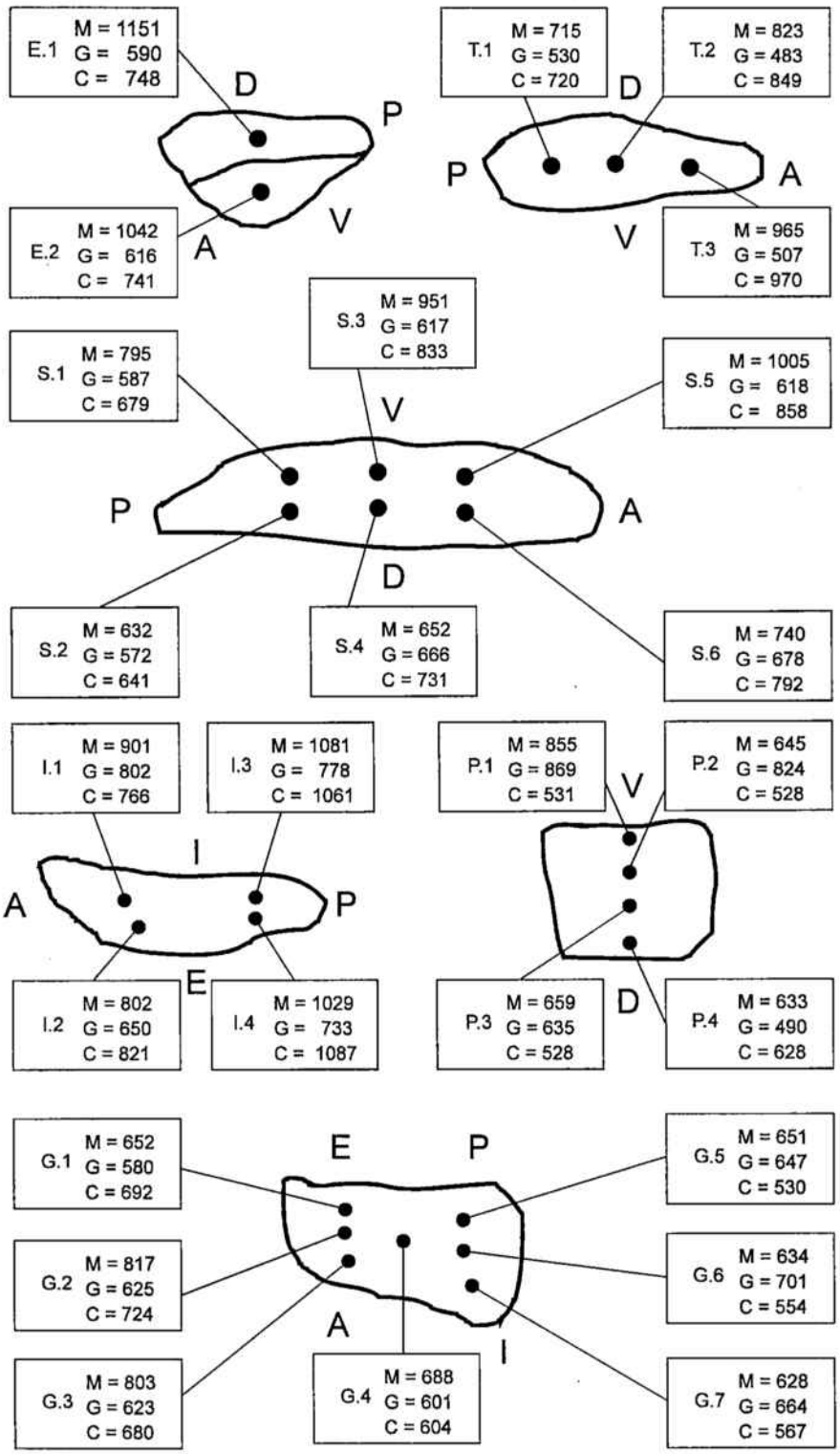


Figure 1. Transversal diagramatic representation of the six muscles studied showing the zones from where fields were chosen for morphometrical measurements. The name of the field (indicated as the initial of the muscle followed by the field number) labels each box, which also shows the total number of fibres measured for each field and for each bird species. *Anatomical location code*: **D**, dorsal; **V**, ventral; **A**, anterior; **P**, posterior; **E**, external; **I**, internal. *Muscles identification code*: **E**, extensor metacarpi radialis; **T**, triceps scapularis; **S**, scapulohumeralis caudalis; **I**, iliotibialis cranialis; **P**, pectoralis; **G**, gastrocnemius lateralis pars externa. *Species identification code*: **M**, mallard duck; **G**, yellow-legged gull; **C**, common coot.

Fibre types and morphometrical measurements.

Fibre types were classified according to the nomenclature of Peter et al. (1972), adapted to bird musculature (see Butler, 1991). In summary, slow oxidative fibres (SO) had a low alkaline and high acidic mATPase stability, a multiple innervation pattern and moderate to high SDH stainings. However, in the deep PEC of the coot these fibres had low SDH stainings and were termed slow white fibres (SW). Fast glycolytic (FG) and fast oxidative glycolytic (FOG) fibres had high alkaline and low acidic mATPase stability and focal innervation. SDH activity was low in FG fibres and high in FOG fibres. Oxidative fibres (SO and FOG) had moderate to high Sudan B staining whilst it was low in the anaerobic fibres (SW and FG).

Fibre type classification and fibre morphometrical measurements for each field were obtained using a light microscope (Dialux, Leitz Wetzlar, Germany) equipped with a camera (Wild MPS51, Heerburg, Switzerland). Fibre measurements were carried out from photomicrographs taken at a magnification of x200 by means of a digitizer tablet (Calcomp 23180-4, Anaheim, CA, USA) connected to a personal computer using suitable software (Sigma-Scan, Jandel Scientific, Erkrath, Germany). A total number of 56,498 fibres were measured. Figure 1 shows the distribution of this number throughout each field and bird species. The number of capillaries in contact with each fibre (NCF), the fibre cross-sectional area (FCSA), the fibre perimeter (FPER) and the maximal oxygen diffusion distance (MDD) represented by the distance between the geometrical center of the fibre and its surrounding capillaries, were measured directly. To obtain these data, at least 40 muscle fibres of each different fibre type per muscle field were randomly selected. In cases where the numbers of a given fibre type were below 40, all the fibres of the field were used to obtain these parameters. Two indices expressing the relationship between the number of capillaries in contact with a fibre and its cross-sectional area ($CCA=NCF\times10^3/FCSA$) or perimeter ($CCP=NCF\times10^2/FPER$) were also calculated. These indices should be seen as a measure of the number of capillaries per 1,000 μm^2 of muscle fibre cross-sectional area (CCA) and the number of capillaries per 100 μm muscle fibre perimeter (CCP).

Statistics

Data from all variables are expressed in the tables as sample mean values with 95% confidence intervals (mean $\pm t_{\alpha,\sigma}$ | α=0.05, σ=4 or σ=6 depending on the species and field). Muscle values (means of field values: Tables 2 to 4) for FCSA, FPER, MDD, NCF, CCA and CCP were analyzed separately for each species and fibre type using a one-way ANOVA and a multiple comparison test by the Scheffé's procedure. Another series of one-way ANOVA and Scheffé's tests, using in this case all the field values, were performed in order to determine between-species differences in the data of the FCSA, FPER, MDD and NCF for each fibre type and muscle. Finally, a cluster analysis using the squared Euclidean distance and the complete method was developed. FCSA, FPER, MDD and NCF were considered as variables after being standardized.

Results

Tables 2, 3 and 4 show the fibre morphometrical measurements (FCSA, FPER, MDD) and the NCF for the mallard, the gull and the coot, respectively. The size of the sample was n=7 in mallards and n=6 in gulls and coots for every field, except for fields S.2, S.4 and S.6 of the mallard where n=6, and for all the TSC muscle of the gull, where n=5. Figures 2 and 3 show a comparative approach for FCSA, FPER, MDD, NCF, CCA and CCP between the three bird species and between the three different fibre types found. The values shown in the histograms are the means of the six muscles studied, which were calculated for each muscle as the average of the field values given in Tables 2, 3 and 4. Beside each histogram a table with the levels of significance from the Scheffé's

Table 2. Fibre values for the mallard duck muscles: fibre cross-sectional area (FCSA), fibre perimeter (FPER), maximal diffusion distance (MDD) and number of capillaries per fibre (NCF). Data are sample means with 95% confidence limits

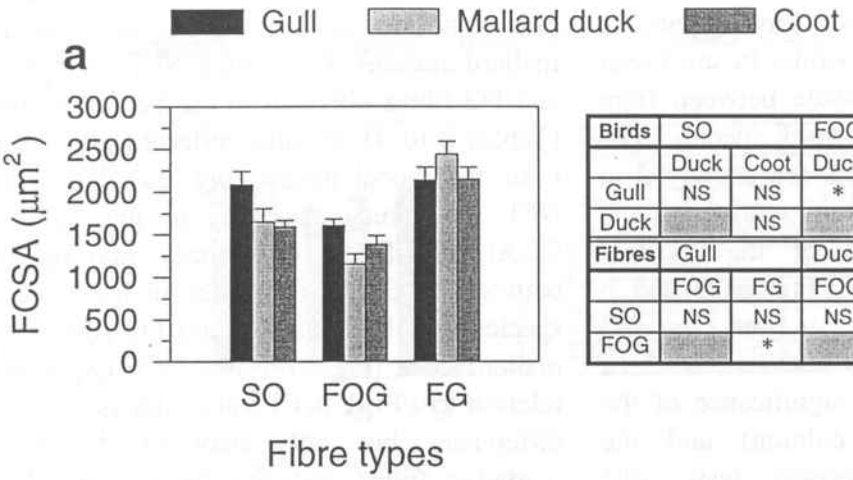
	FCSA			FPER			MDD			NCF		
	SO	FOG	FG	SO	FOG	FG	SO	FOG	FG	SO	FOG	FG
E.1		1002 ± 125	2093 ± 259		119 ± 7	173 ± 12		19.5 ± 1.5	28.1 ± 1.9		3.8 ± 0.3	4.6 ± 0.6
E.2		1014 ± 98	1873 ± 164		119 ± 6	165 ± 8		19.4 ± 1.3	26.6 ± 1.0		3.6 ± 0.4	4.1 ± 0.6
T.1		1061 ± 87	2578 ± 265		123 ± 6	191 ± 11		19.7 ± 1.5	30.4 ± 1.4		3.4 ± 0.2	4.7 ± 0.5
T.2		1052 ± 117	2525 ± 310		121 ± 7	191 ± 13		20.3 ± 1.2	31.3 ± 2.1		3.5 ± 0.3	4.8 ± 0.4
T.3		1082 ± 180	2451 ± 289		123 ± 10	181 ± 10		20.5 ± 2.0	30.5 ± 1.6		3.8 ± 0.4	4.9 ± 0.3
P.1		850 ± 169	2742 ± 378		111 ± 11	201 ± 14		18.4 ± 2.0	33.1 ± 1.8		3.3 ± 0.4	4.3 ± 0.4
P.2		878 ± 115	2584 ± 285		111 ± 8	195 ± 11		18.5 ± 1.7	32.4 ± 1.8		3.3 ± 0.4	4.5 ± 0.4
P.3		773 ± 159	2238 ± 512		103 ± 11	180 ± 20		16.8 ± 2.2	29.7 ± 3.1		3.5 ± 0.4	4.6 ± 0.6
P.4		756 ± 170	1915 ± 327		102 ± 12	166 ± 15		16.7 ± 2.1	26.7 ± 2.4		3.6 ± 0.6	4.7 ± 0.6
S.1		1135 ± 154	2788 ± 500		127 ± 10	200 ± 18		20.8 ± 2.1	32.4 ± 2.6		3.7 ± 0.3	4.6 ± 0.6
S.2		1273 ± 177	3109 ± 460		134 ± 11	213 ± 16		22.5 ± 1.6	35.2 ± 2.9		3.6 ± 0.3	4.9 ± 0.8
S.3	1884 ± 507	1268 ± 220	2678 ± 434	161 ± 21	135 ± 12	199 ± 15	26.7 ± 3.9	22.6 ± 2.5	32.4 ± 2.3	4.6 ± 0.7	4.1 ± 0.3	4.8 ± 0.4
S.4		1210 ± 217	2897 ± 327		131 ± 12	204 ± 13		21.9 ± 1.8	33.6 ± 2.6		3.8 ± 0.5	4.7 ± 0.6
S.5	1743 ± 363	1188 ± 194	2207 ± 309	159 ± 18	131 ± 11	186 ± 15	25.3 ± 2.0	22.0 ± 2.4	29.0 ± 2.0	4.9 ± 0.6	3.9 ± 0.4	4.7 ± 0.4
S.6		1006 ± 133	2458 ± 142		119 ± 9	188 ± 5		19.2 ± 2.5	30.4 ± 1.5		3.3 ± 0.5	4.5 ± 1.0
I.1	1160 ± 118	1094 ± 112	2160 ± 205	130 ± 6	124 ± 7	176 ± 10	20.7 ± 2.8	20.7 ± 1.4	29.0 ± 1.0	3.5 ± 0.2	3.5 ± 0.1	4.2 ± 0.5
I.2		1218 ± 145	2441 ± 391		131 ± 9	186 ± 17		21.5 ± 1.8	30.5 ± 2.3		3.5 ± 0.4	4.2 ± 0.6
I.3	1432 ± 234	1195 ± 192	1925 ± 280	143 ± 11	130 ± 11	169 ± 14	23.0 ± 2.7	21.9 ± 2.0	26.9 ± 2.0	4.8 ± 0.5	4.3 ± 0.4	5.0 ± 0.6
I.4	1405 ± 208	1171 ± 205	1892 ± 245	142 ± 11	130 ± 12	168 ± 12	22.9 ± 2.2	22.0 ± 3.4	26.4 ± 1.1	4.6 ± 0.3	4.8 ± 0.7	4.2 ± 0.3
G.1	2066 ± 157	1767 ± 307	3134 ± 487	171 ± 6	159 ± 16	218 ± 22	29.4 ± 1.8	26.2 ± 3.1	34.5 ± 1.6	5.4 ± 0.5	4.8 ± 0.6	5.5 ± 0.6
G.2	1789 ± 552	1735 ± 312	2639 ± 532	160 ± 25	158 ± 16	200 ± 23	25.7 ± 3.2	25.5 ± 2.1	32.6 ± 3.5	5.9 ± 0.8	5.4 ± 0.6	6.6 ± 0.9
G.3	1911 ± 372	1746 ± 248	2531 ± 449	166 ± 17	159 ± 11	193 ± 19	27.4 ± 2.5	27.1 ± 1.4	31.2 ± 3.2	5.9 ± 0.7	5.9 ± 0.9	6.2 ± 1.3
G.4	1403 ± 103	1614 ± 281	3171 ± 595	143 ± 7	152 ± 13	213 ± 21	23.4 ± 1.7	25.4 ± 2.0	34.4 ± 3.7	5.0 ± 2.3	4.7 ± 0.7	5.7 ± 1.1
G.5		1595 ± 338	3050 ± 603		150 ± 16	209 ± 23		24.8 ± 3.3	33.8 ± 3.4		4.3 ± 0.5	5.2 ± 1.0
G.6		1610 ± 309	3132 ± 518		150 ± 15	211 ± 18		25.0 ± 2.6	33.7 ± 2.1		4.4 ± 0.7	5.3 ± 0.8
G.7		1615 ± 301	3181 ± 578		150 ± 14	213 ± 18		24.5 ± 3.2	34.1 ± 2.7		4.2 ± 0.6	5.3 ± 0.9

Table 3. Fibre values for the yellow-legged muscles: fibre cross-sectional area (FCSA), fibre perimeter (FPER), maximal diffusion distance (MDD) and number of capillaries per fibre (NCF). Data are sample means with 95% confidence limits.

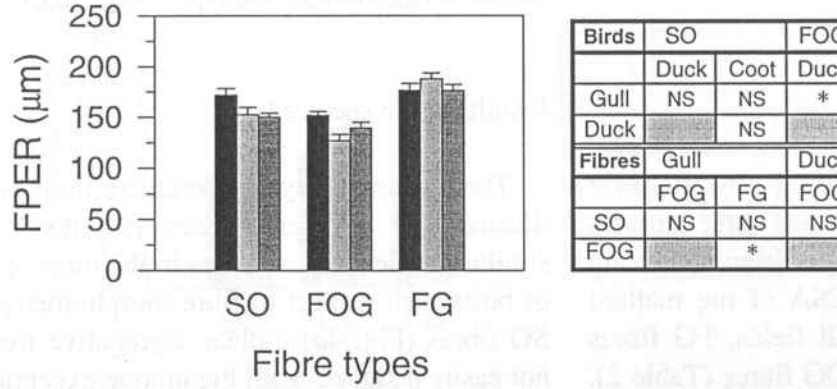
	FCSA			FPER			MDD			NCF		
	SO	FOG	FG	SO	FOG	FG	SO	FOG	FG	SO	FOG	FG
E.1	2553 ± 508	1789 ± 350	2309 ± 516	192 ± 17	161 ± 16	184 ± 22	32.0 ± 2.0	26.1 ± 2.2	30.6 ± 3.3	6.4 ± 1.2	5.8 ± 1.1	6.3 ± 1.3
E.2	1944 ± 233	1840 ± 320	2376 ± 438	170 ± 11	164 ± 13	187 ± 15	28.8 ± 1.5	27.3 ± 2.1	31.6 ± 3.3	5.5 ± 0.9	5.3 ± 1.0	5.8 ± 1.3
T.1		1570 ± 365	2155 ± 608		151 ± 19	178 ± 28		24.6 ± 3.0	28.4 ± 4.0		5.1 ± 1.0	5.4 ± 1.1
T.2		1738 ± 383	2304 ± 355		156 ± 19	179 ± 15		25.0 ± 2.4	28.9 ± 1.7		5.1 ± 0.9	5.5 ± 0.6
T.3		1835 ± 410	2442 ± 563		162 ± 19	188 ± 25		25.9 ± 2.7	30.1 ± 4.0		5.0 ± 0.9	5.6 ± 1.4
P.1		1501 ± 311			147 ± 16			24.4 ± 2.2			5.0 ± 0.5	
P.2		1437 ± 164			143 ± 7			24.0 ± 1.4			4.8 ± 0.5	
P.3		1500 ± 277			146 ± 12			24.3 ± 2.2			4.7 ± 0.6	
P.4		1550 ± 233			148 ± 10			24.2 ± 2.1			5.1 ± 0.7	
S.1		1606 ± 258	2357 ± 492		152 ± 9	184 ± 16		25.5 ± 1.6	29.9 ± 2.4		4.9 ± 0.8	5.5 ± 0.9
S.2		1601 ± 282	2485 ± 422		151 ± 13	189 ± 18		25.3 ± 2.0	30.2 ± 2.8		4.8 ± 0.7	5.6 ± 0.8
S.3	2523 ± 496	1663 ± 340	2396 ± 411	187 ± 18	155 ± 14	187 ± 16	30.3 ± 2.2	25.8 ± 1.7	30.7 ± 1.8	6.0 ± 1.0	5.0 ± 0.7	5.7 ± 1.0
S.4		1580 ± 262	2357 ± 389		152 ± 12	188 ± 17		25.1 ± 2.0	30.6 ± 2.9		4.5 ± 0.4	5.0 ± 0.3
S.5	2336 ± 498	1537 ± 364	2340 ± 385	181 ± 18	150 ± 17	190 ± 15	29.1 ± 2.2	25.2 ± 2.1	31.0 ± 2.6	5.7 ± 1.2	4.8 ± 0.8	5.3 ± 0.8
S.6		1423 ± 184	2206 ± 251		144 ± 10	180 ± 13		24.4 ± 1.7	29.1 ± 1.9		4.5 ± 0.3	5.2 ± 0.5
I.1	1718 ± 271	1284 ± 185	1538 ± 163	154 ± 14	136 ± 9	150 ± 8	25.5 ± 2.1	22.1 ± 1.8	24.2 ± 1.7	4.9 ± 1.1	4.1 ± 0.5	4.4 ± 0.6
I.2	1689 ± 219	1352 ± 202	1609 ± 241	154 ± 13	141 ± 11	152 ± 11	24.3 ± 1.2	22.9 ± 1.9	24.5 ± 2.1	5.1 ± 1.1	4.2 ± 0.3	4.5 ± 0.8
I.3	1852 ± 310	1334 ± 283	1526 ± 186	162 ± 15	140 ± 16	150 ± 11	26.0 ± 2.2	22.7 ± 2.1	24.1 ± 2.3	5.4 ± 0.4	4.3 ± 0.3	4.4 ± 0.5
I.4	1733 ± 256	1305 ± 275	1498 ± 216	156 ± 12	137 ± 15	147 ± 11	25.4 ± 2.2	22.0 ± 2.5	24.3 ± 2.3	5.3 ± 0.6	4.2 ± 0.5	4.6 ± 0.5
G.1	1968 ± 346	1851 ± 128	2347 ± 189	170 ± 15	166 ± 6	191 ± 11	27.8 ± 2.3	27.1 ± 1.0	30.3 ± 1.3	5.4 ± 0.6	5.0 ± 0.6	5.1 ± 0.5
G.2	1853 ± 410	1704 ± 373		163 ± 21	157 ± 19		26.6 ± 2.8	26.1 ± 2.6		5.1 ± 0.9	5.1 ± 0.7	
G.3	2037 ± 459	1748 ± 447		170 ± 21	160 ± 22		27.6 ± 2.5	26.0 ± 2.9		5.4 ± 0.5	5.0 ± 0.6	
G.4	1806 ± 377	1698 ± 303	2151 ± 453	161 ± 19	158 ± 16	178 ± 19	26.4 ± 2.0	25.9 ± 1.5	29.2 ± 2.7	5.0 ± 0.7	4.7 ± 0.5	4.9 ± 0.8
G.5		1711 ± 289	2114 ± 393		157 ± 14	176 ± 17		25.9 ± 1.7	28.4 ± 2.2		4.5 ± 0.4	4.9 ± 0.5
G.6		1623 ± 247	1987 ± 344		153 ± 13	172 ± 15		25.5 ± 0.8	28.1 ± 1.2		4.4 ± 0.4	4.5 ± 0.5
G.7		1684 ± 327	2079 ± 399		157 ± 17	175 ± 18		25.8 ± 2.2	28.5 ± 2.7		4.6 ± 0.6	4.9 ± 0.5

Table 4. Fibre values for the common coot muscles: fibre cross-sectional area (FCSA), fibre perimeter (FPER), maximal diffusion distance (MDD) and number of capillaries per fibre (NCF). Data are sample means with 95% confidence limits

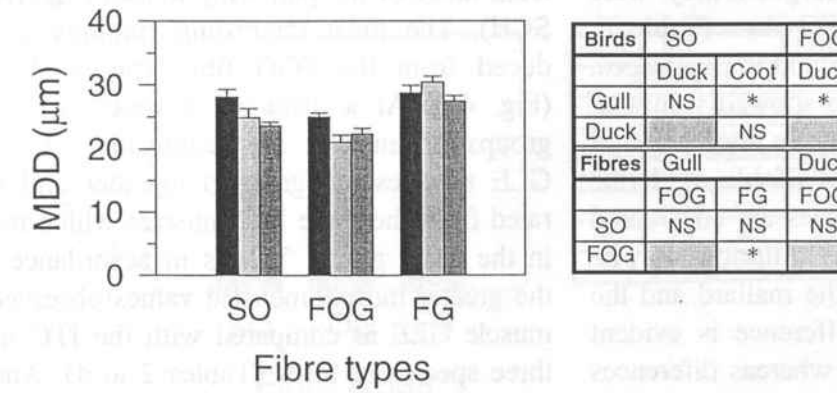
	FCSA			FPER			MDD			NCF		
	SO/SW*	FOG	FG	SO/SW*	FOG	FG	SO/SW*	FOG	FG	SO/SW*	FOG	FG
E.1	1327 ± 250	1224 ± 372	1698 ± 540	139 ± 14	132 ± 19	156 ± 23	22.0 ± 1.7	20.7 ± 1.8	24.8 ± 4.5	5.7 ± 0.6	5.8 ± 0.8	6.0 ± 0.7
E.2	1376 ± 239	1646 ± 320	2008 ± 390	139 ± 12	153 ± 14	173 ± 13	21.4 ± 1.6	24.3 ± 1.5	26.2 ± 1.4	5.5 ± 0.7	6.1 ± 0.7	6.2 ± 0.7
T.1		1378 ± 327	2398 ± 515		140 ± 17	186 ± 21		22.7 ± 3.3	28.9 ± 2.7		4.9 ± 0.3	5.3 ± 0.5
T.2		1349 ± 341	2133 ± 563		134 ± 13	170 ± 21		21.3 ± 1.4	26.0 ± 2.4		5.0 ± 0.8	5.5 ± 0.6
T.3		1242 ± 292	1746 ± 353		130 ± 8	157 ± 14		20.7 ± 1.2	24.7 ± 1.5		4.9 ± 0.4	5.4 ± 0.4
P.1		1073 ± 102			122 ± 6			19.6 ± 1.1			5.0 ± 0.5	
P.2		1069 ± 190			122 ± 11			19.6 ± 1.9			4.8 ± 0.6	
P.3		1030 ± 156			119 ± 9			19.5 ± 1.4			4.6 ± 0.6	
P.4	1608 ± 419*	984 ± 192		148 ± 19*	117 ± 11		23.4 ± 3.2*	18.9 ± 1.9		5.3 ± 0.4*	4.4 ± 0.4	
S.1		1632 ± 205	2612 ± 265		154 ± 10	197 ± 9		24.6 ± 1.4	30.2 ± 1.2		5.0 ± 0.3	5.9 ± 0.2
S.2		1688 ± 129	2354 ± 156		155 ± 6	201 ± 6		24.9 ± 0.9	30.5 ± 1.6		4.9 ± 0.4	5.6 ± 0.5
S.3	1868 ± 311	1462 ± 151	2149 ± 274	165 ± 16	148 ± 8	179 ± 10	25.5 ± 2.8	23.1 ± 1.4	28.5 ± 1.3	5.5 ± 0.5	4.9 ± 0.5	5.5 ± 0.4
S.4		1499 ± 150	2397 ± 282		148 ± 9	187 ± 11		23.3 ± 1.1	29.0 ± 2.0		4.6 ± 0.4	5.5 ± 0.5
S.5	1770 ± 227	1470 ± 123	2080 ± 447	160 ± 10	148 ± 5	175 ± 17	25.4 ± 1.3	23.2 ± 1.2	27.8 ± 2.2	5.6 ± 0.4	5.1 ± 0.3	5.6 ± 0.3
S.6		1339 ± 105	2202 ± 165		139 ± 5	180 ± 7		22.5 ± 1.1	27.4 ± 1.2		4.5 ± 0.5	5.3 ± 0.6
I.1		1316 ± 301	1952 ± 398		134 ± 17	170 ± 18		22.0 ± 2.4	26.7 ± 2.5		4.4 ± 0.3	5.0 ± 0.4
I.2		1240 ± 237	1844 ± 243		132 ± 13	166 ± 12		21.0 ± 2.2	25.7 ± 1.0		4.5 ± 0.4	5.1 ± 0.5
I.3	1657 ± 321	1127 ± 248	1795 ± 466	155 ± 16	129 ± 15	163 ± 19	24.1 ± 1.7	20.7 ± 2.6	25.5 ± 3.3	5.5 ± 0.5	4.7 ± 0.5	5.4 ± 0.7
I.4	1617 ± 375	1112 ± 275	1758 ± 386	151 ± 18	127 ± 16	161 ± 22	23.6 ± 2.0	20.1 ± 2.1	25.8 ± 3.8	5.5 ± 0.6	4.7 ± 0.4	5.4 ± 0.6
G.1	1373 ± 480	1649 ± 455	2595 ± 687	143 ± 25	153 ± 20	193 ± 25	22.3 ± 2.9	24.6 ± 2.3	29.5 ± 2.4	5.0 ± 0.8	5.2 ± 0.8	5.9 ± 0.8
G.2	1477 ± 358	1662 ± 438	2452 ± 724	146 ± 18	155 ± 20	188 ± 26	23.9 ± 2.8	25.2 ± 2.5	29.5 ± 3.0	5.2 ± 0.6	5.4 ± 0.7	5.7 ± 0.6
G.3	1738 ± 467	1769 ± 275	2510 ± 366	158 ± 21	160 ± 12	193 ± 15	25.1 ± 2.7	25.5 ± 1.5	30.5 ± 2.5	5.6 ± 0.9	5.4 ± 1.0	5.9 ± 0.8
G.4		1825 ± 371	2549 ± 405		160 ± 16	194 ± 15		26.3 ± 2.3	30.2 ± 2.6		5.2 ± 0.6	5.6 ± 0.6
G.5		1867 ± 343	2764 ± 514		161 ± 15	200 ± 19		26.6 ± 2.1	30.9 ± 2.7		5.1 ± 0.5	5.4 ± 0.5
G.6		1809 ± 127	2567 ± 187		160 ± 5	193 ± 7		25.9 ± 0.7	30.6 ± 1.9		5.3 ± 0.5	5.3 ± 0.5
G.7		1897 ± 353	2597 ± 396		163 ± 15	194 ± 15		26.8 ± 3.0	30.5 ± 3.6		5.2 ± 0.5	5.2 ± 0.5



Birds	SO		FOG		FG	
	Duck	Coot	Duck	Coot	Duck	Coot
Gull	NS	NS	*	NS	NS	NS
Duck		NS		NS		NS
Fibres	Gull		Duck		Coot	
	FOG	FG	FOG	FG	FOG	FG
SO	NS	NS	NS	*	NS	*
FOG		*		***		**



Birds	SO		FOG		FG	
	Duck	Coot	Duck	Coot	Duck	Coot
Gull	NS	NS	*	NS	NS	NS
Duck		NS		NS		NS
Fibres	Gull		Duck		Coot	
	FOG	FG	FOG	FG	FOG	FG
SO	NS	NS	NS	*	NS	*
FOG		*		***		**



Birds	SO		FOG		FG	
	Duck	Coot	Duck	Coot	Duck	Coot
Gull	NS	*	*	NS	NS	NS
Duck		NS		NS		NS
Fibres	Gull		Duck		Coot	
	FOG	FG	FOG	FG	FOG	FG
SO	NS	NS	NS	*	NS	NS
FOG		*		***		**

Figure 2. Histograms showing the mean of the six muscles studied, calculated for each muscle as the average of the field values, for the following parameters: *a*, fibre cross-sectional area (FCSA); *b*, fibre perimeter (FPER); *c*, maximal diffusion distance (MDD). Bars represent the standard error of the mean. Tables show the levels of significance after running Scheffé's multiple comparison tests. In the upper part of each table (**Birds**) species comparisons are tested regarding each fibre type. The lower part of each table (**Fibres**) shows the results of comparing fibre types with regard to each species. *Significance values*: ns = not significant; * = $0.05 \geq p > 0.01$; ** = $0.01 \geq p > 0.001$; *** = $p \leq 0.001$.

multiple comparison test is displayed. The upper half of each table compares bird species in respect of each fibre type, whilst in the lower half the comparisons are made between fibre types with regard to each bird species. The results of the cluster analysis are displayed in Figure 4. Each dendrogram represents the affinities and segregations of the species-muscles for each fibre type. Figures 5 and 6 show one small table for each fibre type and muscle, with the differences between the three bird species. The levels of significance of the one-way ANOVA (first column) and the Scheffé's multiple comparison tests (the remaining columns) for each parameter are given.

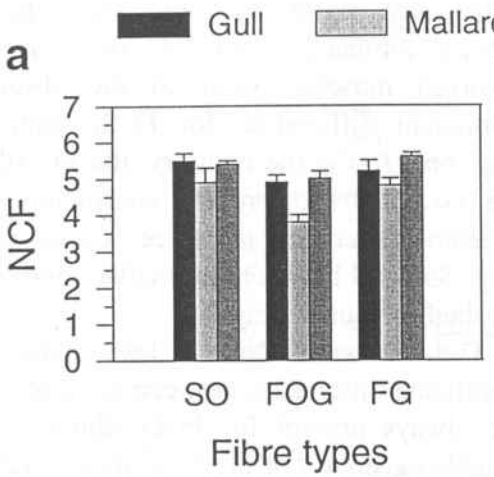
Results at the fibre type level

The measurements of FCSA, FPER and MDD for the FOG fibres were always lower than for the FG fibres in the three species studied (Tables 2 to 4); this difference being specially notable for the FCSA of the mallard muscles where, in almost all fields, FG fibres were two-fold larger than FOG fibres (Table 2). These variables presented intermediate values for SO fibres in mallard and coot muscles (Tables 2 and 4), whilst in gulls they were similar to those found in the FG fibres (Table 3). The unusual SW fibre type found in the deepest part of the coot PEC muscle showed values of FCSA, FPER and MDD within the range of those found in the SO fibres (Table 4). If the global means of all the muscles are considered (Fig. 2) these differences and similarities may be statistically confirmed. For the mallard and the coot, a slight significant difference is evident between SO and FG fibres, whereas diferences are highly significant between FOG and FG fibres and not significant between SO and FOG. The morphometrical parameters are more similar between the three fibre types of the gull, where the statistical difference is minimal for the pair FOG-FG and is not significant for the other pairs.

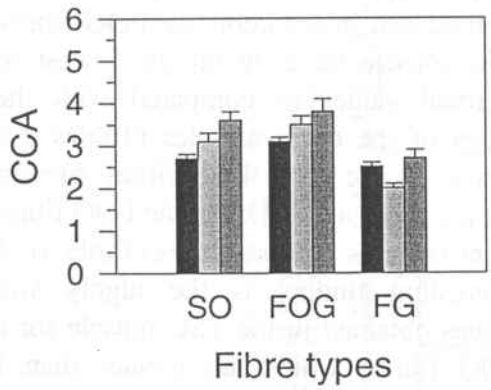
The results from Tables 2 to 4 also present lower NCF for FOG than for FG fibres although, if global means are considered (Fig. 3*a*), significant diferences are only found for mallard muscles. The similar NCF for SO, FOG and FG fibres obtained in all the fields studied (Tables 2 to 4) are also reflected after considering the global means (Fig. 3*a*). However, if NCF is expressed relative to the FCSA (as CCA), significant differences may be seen between FOG and FG fibres in the three bird species, and between SO and FG fibres in the mallard duck (Fig. 2*b*). The NCF expressed in relation to FPER (CCP) also shows significant differences but only between the mallard oxidative fibres and FG fibres (Fig. 2*c*). A common feature of the three species is the lack of significant differences in both indices between SO and FOG fibre types (Fig. 3*b*,*c*).

Results at the species level

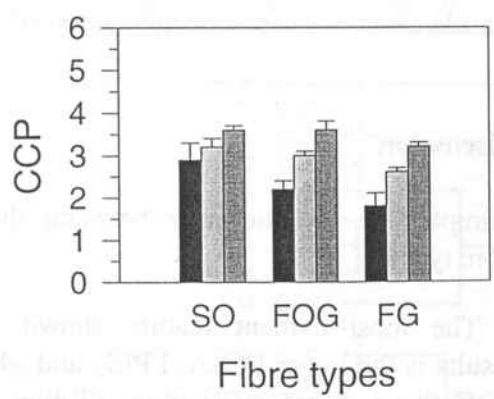
The cluster analysis presented in Figure 4 illustrates a number of key features of the similarities between muscles in the three species of birds with respect to fibre morphometry. For SO fibres (Fig. 4*a*) a clear segregative trend is not easily deduced, with the unique exception of the SO fibres from the gull leg muscles (ITC and GLE), which are relatively separated (d=13) from those of the gull wing muscles (EMR and SCH). The most interesting findings are deduced from the FOG fibre-type dendrogram (Fig. 4*b*). At a distance of d=43 two major groups of muscles are segregated. The three GLE muscles are grouped together and separated from the three ITC muscles which remain in the other group. This is in accordance with the greater morphometrical values observed for muscle GLE as compared with the ITC in the three species of birds (Tables 2 to 4). Another main finding is the fact that, with the exception of the ITC muscle, all the muscles of the gull are placed in a distinct group from that of the mallard muscles (except GLE). This distribution results in a perfect grouping of the mallard muscles involved in aerial locomotion, on the one hand, and those of the gull, on the other. In contrast, the flight muscles of the coot are scattered throughout the two main groups.



Birds	SO		FOG		FG	
	Duck	Coot	Duck	Coot	Duck	Coot
Gull	NS	NS	**	NS	NS	NS
Duck		NS		**		NS
Fibres	Gull		Duck		Coot	
	FOG	FG	FOG	FG	FOG	FG
SO	NS	NS	NS	NS	NS	NS
FOG		NS		*		NS



Birds	SO		FOG		FG	
	Duck	Coot	Duck	Coot	Duck	Coot
Gull	NS	*	NS	*	NS	NS
Duck		NS		NS		**
Fibres	Gull		Duck		Coot	
	FOG	FG	FOG	FG	FOG	FG
SO	NS	NS	NS	**	NS	NS
FOG		**		***		*



Birds	SO		FOG		FG	
	Duck	Coot	Duck	Coot	Duck	Coot
Gull	NS	NS	**	***	*	***
Duck		NS		*		NS
Fibres	Gull		Duck		Coot	
	FOG	FG	FOG	FG	FOG	FG
SO	NS	NS	NS	***	NS	NS
FOG		NS		***		NS

Figure 3. Histograms showing the mean of the six muscles studied, calculated for each muscle as the average of the field values, for the following parameters: *a*, number of capillaries per fibre (NCF); *b*, number of capillaries in relation to the fibre cross-sectional area (CCA = NCF×10^3/FCSA); *c*, number of capillaries in relation to the fibre perimeter (CCP = NCF×10^2/FPER). Bars represent the standard error of the mean. Tables show the levels of significance after running Scheffé's multiple comparison tests. In the upper part of each table (**Birds**) species comparisons are tested regarding each fibre type. The lower part of each table (**Fibres**) shows the results of comparing fibre types with regard to each species. *Significance values*: ns = not significant; * = $0.05 \geq p > 0.01$; ** = $0.01 \geq p > 0.001$; *** = $p \leq 0.001$.

Figure 4*b* also shows that, although a certain segregation is found between the PEC and TSC muscles of the mallard and the coot (d=13), they are in the same great group, far from the PEC and TSC muscles of the gull (d=43). The SCH muscles of the coot and the gull are placed together and segregated from the mallard SCH muscle. For the FG fibres, the three ITC muscles are placed in the same great group but relatively segregated (the gull from the mallard-coot at d=11 and the mallard from the coot at d=10, Fig. 4*c*). With regard to the GLE muscles, the mallard and the coot are placed in the same great group highly segregated from the gull (d=51, Fig. 4*c*). Almost all the wing muscles of the mallard and the gull are grouped together in two different but closer groups, whereas those of the coot wing are dispersed.

Tables 2 to 4 show lower values for the mallard than for the other two species studied in all the parameters of the wing muscles (EMR, TSC, SCH and PEC), whilst similar values for the leg muscles (ITC and GLE) are found in the three species. However, when global means from all muscles are considered, it can be seen that these differences are significant only between the gull and the mallard for the FOG fibres and in MDD of SO fibres for the pair gull-coot (Fig. 2). If the number of capillaries per FCSA and FPER are considered (CCA and CCP), a general trend for these indices is clear (Fig. 3*b*,*c*): both have, for the oxidative fibres (SO and FOG), the lowest value for the gull muscles, an intermediate value for the mallard and the highest for the coot. These differences in CCA are significant between the gull and the coot for SO and FOG fibres; and between the mallard and the coot for FG fibres. In CCP they are significant for all the pairs of species regarding the FOG fibres and between the gull and the other two species for FG fibres (Fig. 3*c*).

Figures 5 and 6 statistically analyze in more detail the morphometrical values shown in Tables 2 to 4. SO fibres have similar measurements in the GLE muscle (Fig. 5*a*) but some differences are noted between the mallard and the other species for the ITC muscle (Fig. 5*d*). With regard to the SCH muscle, it is interesting to note that the higher morphometrical values found for the SO fibres of the gull (Table 3) are statistically significant (Fig. 6*a*). Between-species comparisons for FG fibres in aerial-involved muscles result in the absence of significant differences for FCSA and FPER (Fig. 6*b*,*d*,*f*). On the contrary, the FG fibres of the GLE muscle present statistically strong differences between the three species of birds (Fig. 5*b*), and between the mallard and the gull for the ITC muscle (Fig. 5*e*).

The one-way ANOVA tests indicate that significant differences between the three species are always present for FOG fibres, with the notable exception of the GLE muscle (Fig. 5*c*). The high significance of the differences in the morphological parameters of PEC muscles for all pairs (Fig. 6*h*) must be pointed out. In the mallard and in the coot, the FOG fibres of the PEC muscle have by far the lowest morphometrical values as compared with the FOG fibres of the other muscles (Tables 2 and 4), whilst in the gull these fibres present comparable sizes and MDD to the FOG fibres of the other muscles of this species (Table 3). Another interesting finding is the highly significant values obtained in the TSC muscle for the gull FOG fibres, which are greater than for the mallard and the coot (Fig. 6*e*, Tables 2 and 3). The strong statistical difference between the mallard and the other two species in the SCH muscle also deserves to be mentioned (Fig. 6*c*).

Discussion

Comparative morphometry between the three fibre types

The most evident feature shown by our results is the lower FCSA, FPER and MDD for FOG fibres than for FG fibres (Tables 2 to 4; Fig. 2). This is a common trend, not only in avian musculature (see George & Berger, 1966) but also in skeletal muscles of vertebrate species as different as reptilian or mammalian, and with body weights ranging from those of rats to horses (Gleeson et al. 1984; Sullivan & Pittman, 1987; Ishihara et al. 1991; Laidlaw et al. 1995; Rivero et al. 1996). Since the final stage in

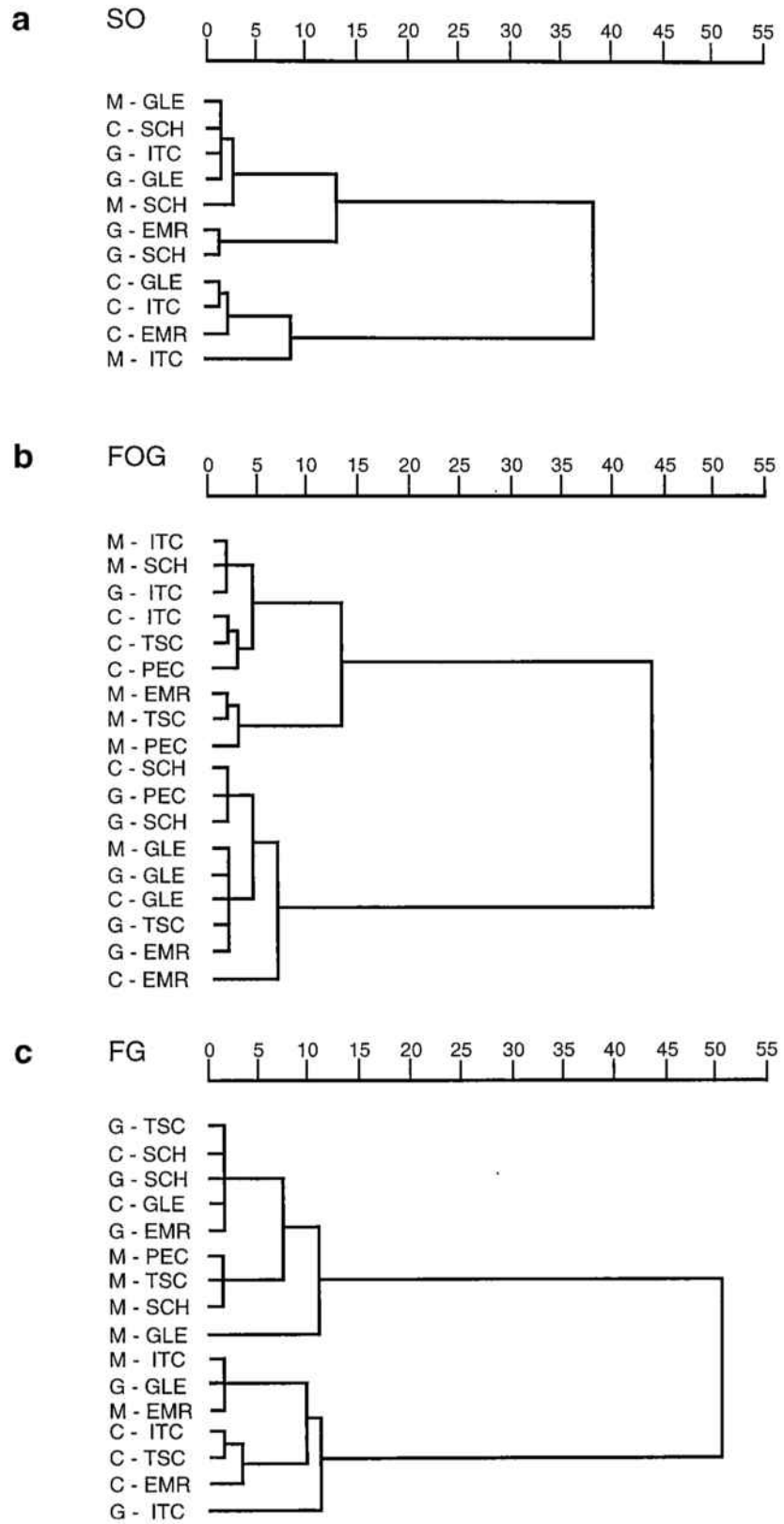


Figure 4. Cluster analysis of all the species-muscles by using the squared Euclidean distance and the complete method: *a*, for SO fibre-type; *b*, for FOG fibre-type; *c*, for FG fibre-type. On the left, the initial of the common English name of each bird species (*M*, mallard duck; *G*, yellow-legged gull; *C*, common coot) is followed by a hyphen and the abbreviature of each muscle (see Table 1).

oxygen delivery is transport from the capillaries to the mitochondria, the lower values for FOG and SO fibres, in mean fibre size and diffusion distances, is a consequence of their higher rate of oxidative metabolism. The fact that SO fibres in the three bird species studied had a moderate SDH staining intensity, as compared to the high or very high staining of the FOG fibres (see Material and Methods and Torrella et al. 1996), may explain the low statistical differences between SO and FG fibres for the morphometric parameters (Fig. 2). It is possible that the greater surface area to volume ratios of the smaller fibres may influence their metabolic functions, as proposed Gleeson & Harrison (1988), who found an inverse relationship between FCSA and fibre enzyme activities in reptilian muscle.

In contrast to what had been found in some reports (Romanul, 1965; Gray & Renkin, 1978), where the fibres with a high oxidative capacity had a greater NCF than those with anaerobic metabolism, it can be deduced from our results that the oxidative differences between FOG, SO and FG fibre-types did not match a similar difference in the NCF (Fig. 3*a*). Such findings agree with those reported for several muscles of five mammalian species with a wide range of muscle fibre size (Plyley & Groom, 1975); and with Mathieu-Costello et al. (1992), who reported not significant differences in the NCF between rat soleus and hummingbird PEC muscle at a given mitochondrial volume, in spite of their very unlike values in FCSA and FPER. Moreover, our results show that the differences in oxidative capacity between FOG and FG fibres are accompained by differences in CCA for the three bird species (Fig. 3*b*) and in CCP for the mallard muscles (Fig. 3*c*). This is consistent with the speculation that the increment in oxygen supply, needed by the oxidative fibres, may be achieved by decreasing the size of the muscle fibre rather than by increasing the NCF (Plyley & Groom, 1975; Sullivan & Pittman, 1987).

Comparative morphometry between the three species

General fibre morphometry

When global muscle means are considered, the resemblance in fibre morphometry between the three bird species is surprising. Statistical differences in all the morphometrical variables were only found between the mallard and the gull for FOG, and in MDD between the gull and the coot for SO fibres (Fig. 2). This fact may indicate that, considering the animal as a whole, the general morphometry of the skeletal muscle fibres is a conservative feature unrelated to life style, at least among these three species of birds. By contrast, if the muscles are considered individually, the different habits of locomotion between these three species result in considerable phenotypic variation, as is reflected in Figures 5 and 6, and discussed in the next section. When CCA and CCP are considered, a general trend in relation to the different locomotor performances may be seen after the histograms (Fig. 3*b*,*c*). The finding that for the oxidative fibres (SO and FOG) both indices present values in the order gull<mallard<coot matches the energetics of the different locomotor habits performed by these birds. Gulls predominantly glide during flight whilst mallards and coots are flapping fliers (Del Hoyo et al. 1992; 1996). The evidence that flapping flight involves higher aerobic energetic demands than gliding (Baudinette & Schmidt-Nielsen, 1974; Goldspink et al. 1977) and the fact that mallards and coots are more active swimmers than gulls, may account for the lower CCA and CCP indices found in the gull. The higher values for the coot than for the mallard are likely to be a consequence of their feeding habits: coots frequently perform short dives when feeding whereas mallards usually only submerge their head (Del Hoyo et al. 1992; 1996). Since short dives demand a significant aerobic effort (Butler, 1991), the oxidative muscle fibres of the coot require greater CCA and CCP than those of the mallard.

It is notable that these differences found between the three species show greater statistical significances in FOG fibres for CCP

a GLE

SO	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	NS	NS	NS	NS
FPER	NS	NS	NS	NS
MDD	NS	NS	NS	NS
NCF	NS	NS	NS	NS

b GLE

FG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	***	**	***	**
FPER	***	**	***	**
MDD	***	***	***	NS
NCF	**	NS	**	*

c GLE

FOG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	NS	NS	NS	NS
FPER	NS	NS	NS	NS
MDD	NS	NS	NS	NS
NCF	NS	NS	NS	NS

d ITC

SO	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	**	*	**	NS
FPER	**	*	**	NS
MDD	*	NS	*	NS
NCF	*	NS	NS	NS

e ITC

FG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	**	NS	**	NS
FPER	***	NS	***	*
MDD	**	NS	**	NS
NCF	**	*	NS	*

f ITC

FOG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	*	NS	*	NS
FPER	**	NS	**	*
MDD	*	NS	NS	*
NCF	*	*	NS	NS

Figure 5. One-way ANOVA and Scheffé's multiple comparison tests for the muscles of the leg. Each table shows, in the first column, the levels of significance of the one-way ANOVA test. Inside the cells of the remaining columns the results of the multiple comparison tests for the fibre cross-sectional area (FCSA), fibre perimeter (FPER), maximal diffusion distance (MDD) and number of capillaries per fibre (NCF) are given. *a-c*: SO, FG and FOG fibres of the gastrocnemius muscle (GLE); *d-f*: SO, FG and FOG fibres of the muscle iliotibialis cranialis (ITC). *Significance values*: ns = not significant; * = $0.05 \geq p > 0.01$; ** = $0.01 \geq p > 0.001$; *** = $p \leq 0.001$.

than for CCA (Fig. 3), which is indicative of the important functional role that the capillary distribution per unit of FPER has in blood-tissue oxygen delivery from capillaries to mitochondria. This is consistent with the findings on subsarcolemnal mitochondrial distributions (Weibel, 1984; Swatland, 1985; Suarez, 1992); with the evidence that the regulated parameter in capillary network growth may be the number of capillaries per muscle fibre perimeter unit (Snyder, 1995); and with the suggestion that the size of the capillary-to-fibre perimeter interface is one of the main determinants of oxygen flux rate (Mathieu-Costello, 1993).

SO fibres

The presence of SO fibres with greater sizes in the SCH and EMR muscles of the gull than those of the mallard or the coot (Tables 3 and 4, Fig. 6*a*), is attributed to the mechanical function of these fibres during flight. The SCH muscle of gulls contributes to maintaining the humerus outstretched and the EMR to extending the wrist during gliding (Meyers, 1992; 1993). This role of the SO fibres of the forelimb muscles implies a more constant and greater use of these fibres in the gull than in the other two species. Since muscle fibre size is known to be related to muscle use, resistance training increasing and inactivity decreasing the FCSA (Nicks et al.

a SCH

SO	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	*	NS	*	*
FPER	*	NS	*	*
MDD	*	NS	*	*
NCF	*	NS	*	NS

b SCH

FG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	NS	NS	NS	NS
FPER	*	NS	NS	NS
MDD	**	**	NS	NS
NCF	***	***	***	NS

c SCH

FOG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	***	***	***	NS
FPER	***	***	***	NS
MDD	***	**	***	*
NCF	***	***	***	NS

d TSC

FG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	NS	NS	NS	NS
FPER	NS	NS	NS	NS
MDD	*	*	NS	NS
NCF	***	***	***	NS

e TSC

FOG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	***	*	***	**
FPER	***	*	***	**
MDD	***	NS	***	**
NCF	***	***	***	NS

f EMR

FG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	NS	NS	NS	NS
FPER	NS	NS	NS	NS
MDD	*	NS	NS	*
NCF	*	*	*	NS

g EMR

FOG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	*	NS	*	NS
FPER	*	NS	*	NS
MDD	*	NS	*	NS
NCF	**	**	*	NS

h PEC

FOG	ANOVA	Duck vs Coot	Duck vs Gull	Coot vs Gull
FCSA	***	***	***	***
FPER	***	**	***	***
MDD	***	**	***	***
NCF	***	***	***	NS

Figure 6. One-way ANOVA and Scheffé's multiple comparison tests for the muscles involved in aerial locomotion. Each table shows, in the first column, the levels of significance of the one-way ANOVA test. Inside the cells of the remaining columns the results of the multiple comparison tests for the fibre cross-sectional area (FCSA), fibre perimeter (FPER), maximal diffusion distance (MDD) and number of capillaries per fibre (NCF) are given. *a-c*: SO, FG and FOG fibres of the scapulohumeralis muscle (SCH); *d-e*: FG and FOG fibres of the muscle triceps scapularis (TSC); *f-g*: FG and FOG fibres of the muscle extensor metacarpi radialis (EMR); *h*: FOG fibres of the muscle pectoralis (PEC). *Significance values*: ns = not significant; * = $0.05 \geq p > 0.01$; ** = $0.01 \geq p > 0.001$; *** = $p \leq 0.001$.

1989; López-Rivero et al. 1992), the greater size found for SCH and EMR muscles of the gull could have been selected in order to match these greater activity needs. Moreover, the isometric tension, for which SO fibres are especialized (Goldspink, 1981), may be better developed by fibres with a greater cross-section than by narrow ones, since they can generate more tension (Lucas et al. 1987; Elzinga et al. 1989). The segregation found in the dendrogram for these fibres between the gull muscles of the wing and the leg (Fig. 4*a*), is also seen as a result of this locomotor contribution of the SO fibres to gliding flight. With regard to the SO fibres of the leg muscles, the absent or weak statistical differences between the three species (Fig. 5*a,d*) may be indicative of the similar functional role in maintaining the posture attributed to these fibres in the leg (Suzuki et al. 1985).

FOG fibres of the forelimb muscles

In the PEC muscles, highly significant size differences for FOG fibres between the three species are found. Tables 2 to 4 show that the highest values in FCSA, FPER and MDD are measured for the gull and the lowest for the mallard PEC. Taking the FCSA as an example, the FOG fibres of the gull PEC muscle have a cross-section which averages 1.8 and 1.4 times that of the mallard and the coot, respectively. These values result in the highly significant differences found between the PEC muscles of these species (Fig. 6*h*), and justify the segregation of the PEC of the mallard and the coot from that of the gull observed in the cluster analysis (Fig. 4*b*). The PEC muscle is responsible for depressing the wing during flapping flight and its function is also preponderant in maintaining the wings outstretched during gliding (Table 1). The differences in energetic demands between flapping and gliding modes of flight impose several adaptations on bird's physiology (Butler & Woakes, 1980; Meyers, 1993), among which the morphometry of skeletal muscle oxidative fibres should not be an exception. Since the tension that a muscle fibre can develop is proportional to its cross-sectional area (Lucas et al. 1987; Elzinga et al. 1989), the muscular architecture of the FOG fibres of the gull PEC, with greater FCSA and FPER, is suited to afford the long-lasting activity subjected to the great stress of tension required during gliding. It also corresponds to the low metabolic demands (Baudinette & Schmidt-Nielsen, 1974; Goldspink et al. 1977) and to a relatively low beating rate (2.8 s^{-1}) of the gull wings (Meinertzhagen, 1955). By contrast, the FOG fibres of the mallard and the coot PEC muscles attain their high oxidative function, required by sustained flapping flight and wing beat frequencies of 5.0 s^{-1} (mallard) and 5.8 s^{-1} (coot) (Meinertzhagen, 1955), by reducing its size at the expense of a disminished ability for force generation. This small fibre size has a functional importance inasmuch as it reduces not only the MDD for the oxygen, but also the transport distances for the metabolites and other substrates.

Several other examples may be found in the literature where the small fibre size seems a very effective way to improve the oxidative capacity and the fatigue resistance of the muscle fibres. Lundgren & Kiessling (1988) found smaller FCSA in species of passerine birds with long-distance migratory behaviours (785 μm^2) than in passerines which cover short-distance migrations (981 μm^2) or partial migrators (1,176 μm^2). León-Velarde et al. (1993) obtained, in the FOG fibres of the PEC muscle of the Andean coot (*Fulica americana peruviana*), FCSA and NCF values (1,055 μm^2 and 4.8) almost identical to those we have measured in the common coot (Table 4). The close phylogenetic relationship between the two species of coots and, more importantly, the almost identical mode of flight, may explain the similarities of these data. In the pigeon, which is a very active flapping flier, Viscor et al. (1992) reported FCSA for FOG fibres ranging from 600-700 μm^2 in the PEC muscle, while Mathieu-Costello (1991) and Mathieu-Costello et al. (1994) found even lower fibre size values (304-782 μm^2 and 186-468 μm^2). Calculated from the fibre diameters or from the fibre densities given

by George & Naik (1957), Kaiser & George (1973) and Rakušan et al. (1971), the aerobic fibres of pigeon PEC muscle also presented lower circular FCSA (855 μm^2, 219-633 μm^2 and 500-600 μm^2) than those reported here for either the mallard or the coot (Table 2). An extreme example is seen in the PEC muscles of the hummingbirds, which hover to feed. During hovering flight wing beat frequencies of up to 78 s^{-1} have been recorded (Greenewalt, 1962). This imposes numerous physiological constraints on hummingbirds (Suarez, 1992) which, at the level of muscle morphometry, are accomplished by mean FCSA values of 201 μm^2, among other features (Mathieu-Costello, 1993). In aquatic species, Turner & Butler (1988) found a mean FCSA value of 389 μm^2 in FOG fibres of the PEC muscle of a diving bird (the tufted duck), which is considerably lower than our findings for the mallard (Table 2). In this case, the morphological and physiological constraints derived from diving versus dabbling behaviour of the tufted and the mallard duck may explain these differences (Keijer & Butler, 1982; Torrella et al. 1996)

The significantly higher morphometrical values found in the gull than in the other two species for the FOG fibres of the TSC muscle (Table 3, Fig. 6*e*), and the fact that the TSC of the mallard and the coot (for FOG fibres) are in the same great group which segregates from the TSC of the gull (d=43, Fig. 4*b*), can be also explained by the argument proposed for the PEC muscle. In order to pull the tendons that maintain the humerus extended during gliding, gulls may need broader fibres in their TSC muscles capable of developing greater tension than the narrow ones, which are required by ducks and coots to extend and stabilize the elbow during flapping flight.

FOG fibres of the hindlimb muscles

The results from Tables 2 to 4 show that, for the three species, the morphometric values found for the ITC muscle are much lower than those for the three GLE muscles. Moreover, the cluster analysis places all the three ITC muscles together in a different group at a great distance (d=43, Fig. 4*b*) from that of the three GLE muscles. The different mechanical and functional roles of both muscles during swimming may explain this grouping. The affinities between the three GLE muscles, on the one hand, and the three ITC muscles, on the other, may be also deduced from the Figure 5*c,f*, where no level of significance was detected for the FOG. The fact that the three species studied here are in some way aquatic birds, which need to properly manage sustained swimming abilities, may result in an equivalent or very similar FOG fibre morphometry in the three GLE and ITC muscles. This indicates that the compromise between an adequate fibre oxygen flux rate and the development of power, to overcome the water's drag force, should be highly optimized. During swimming, the tarsometatarsus acts as a lever (Sigmund, 1959) exerting force against the water with the lobed feet (in coots) or webbed ones (in ducks and gulls). So that the maximum surface area will be available for the power stroke the toes are totally extended, whereas they are folded during the recovery phase to reduce drag forces (Storer, 1960; Clark & Fish, 1994). Thus, the GLE muscle, which is responsible for the extension of the tarsometatarsus (Table 1), must provide great power to overcome the drag force of the water; while the ITC muscle, which protracts the femur (Table 1) during the recovery phase, needs to develop less power. Moreover, Sigmund (1959) found that, in coots during swimming, the ankle joint has greater mobility (a total angle of 86°) than the knee joint (with only an angle of 23°) due to the considerable enlargement of the cresta patellaris. This enlargement in the tibiofemoral articulation allows the insertion of some muscular groups such as the Mm. iliotibiales, Mm. femorales, the extensor digitorum longus and the tibialis anterior muscles (George & Berger, 1966; Baumel, 1979), and is considerably developed in certain aquatic forms as an adaptation to swimming and diving (Bellairs & Jenkin, 1960). Thus, the differences both in power requirements and in the mobility angle of the ankle joint, can explain the great contrasts in fibre morphometry between the FOG fibres of the GLE and ITC muscles.

FG fibres

The FG fibres of the three species have very close morphometric characteristics in the muscles involved in aerial locomotion (Fig. 6*b*,*d*,*f*), but present important differences for the leg muscles, especially for the GLE. These fibres have significantly greater sizes in the GLE muscle of the mallard and the coot than of the gull (Fig. 5*b*), while the difference is only present between the mallard and the gull for the ITC muscle (Fig. 5*e*). These results are reflected by the cluster analysis, where FG fibres of muscles GLE of the mallard and the coot are joined together and segregated from the GLE of the gull (Fig. 4*c*). FG fibres are mainly recruited during short bursts of activity, when high power output requirements are needed (Alexander & Goldspink, 1977). All the muscles studied here are somehow involved in these kind of activities: the pectoral limb muscles, especially during the take-off or landing, and the leg muscles during the sprint locomotion (either terrestrial or swimming). In spite of this, the fibre morphometric profile of FG fibres only presents significant differences between the three species of birds for the GLE muscle, the most massive of the lower leg. The differences in drag forces, which muscles must overcome to generate locomotion, between aerial and aquatical environments should be considered to explain these findings. It is also possible that, for aerial locomotion, the differences in high muscle power requirements between these birds would be regulated by the amount (percentage) of FG fibres. In fact, these fibres were totally absent in the PEC muscles of the coot and the gull, while the FG fibres with considerable FCSA (Table 2) have a notable presence in the superficial parts of the mallard PEC muscle (Torrella et al. 1996). This bird shows a marked pattern of vertical and explosive take-off for which these fibres may be useful, as has been proposed for pigeon PEC (Rosser & George, 1986) where FG fibres of similar size have also been found (Viscor et al. 1992; Mathieu-Costello et al. 1994).

Conversely, in the leg muscles and especially in the GLE, not only the numbers of FG fibres but also the FCSA and FPER would play an important role in performing high power foot strokes. In sharp contrast to what has been said for the oxidative fibres, the FG fibres have fewer size restrictions because of their relative independence from oxygen supply. This will enable the FG fibres to become large enough to provide the mallard and the coot GLE muscles with rapid, powerful and short-duration contractions. Activities such as sprint swimming or taking-off from water either by a single thrust of both feet, as in the mallard (Del Hoyo et al. 1992), or by short runs over the water, as is the case of the coot (Rüppell, 1977), will therefore be easily achieved. An additional argument in favour of this is the report of great FG fibre sizes (4,662 μm^2) in muscovy ducklings *Cairina moschata* (Duchamp et al. 1992). The main method of locomotion and escaping from predators for ducklings is burst swimming, since they are unable to fly until 49-60 days (see Aigeldinger & Fish, 1995). These authors found that the feet of ducklings must generate not only thrust but also lift to be able to perform hydroplaning, a mechanism of motion which achieves high surface speeds (Aigeldinger & Fish, 1995). Therefore, in order to attain the powerful contractions needed to lift the bird, FG fibres with great FCSA (more than 50% greater than our figures) may be useful and effectively recruited. In adult ducks, other studies have reported FCSA values which are much the same as our results for the mallard and the coot. Turner & Butler (1988) measured FG fibres with a FCSA of 1,691 μm^2 in the red and 2,382 μm^2 in the white GLE muscle of the tufted duck (*Aythya fuligula*) and Snyder (1990) found, considering together the FOG and FG fibres, a mean value of 2,000 μm^2 in the GLE muscle of mallard.

Since between-species comparisons of the fibre morphometric values for the GLE muscle only yielded statistical differences for the FG fibres (Fig. 5*a*,*b*,*c*), and considering that the GLE muscle is responsible for paddling during swimming (Table 1), it seems reasonable to hypothesize that the morphometrical differences in FG fibres of this muscle are an important adaptative response to force generation, as a response to the different locomotory behaviours of each species.

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