Int. J. Dev. Biol. 42: 653-661 (1998)

*Original Article*

# Expression of myosin heavy chain isoforms during development of domestic pigeon pectoralis muscle

BENJAMIN W.C. ROSSER[1*], MACDONALD WICK[2#], DONNA M. WALDBILLIG[1], DEBRA-JANE WRIGHT[1], CINDY M. FARRAR[1] and EVERETT BANDMAN[2]

[1]*University of Saskatchewan, College of Medicine, Department of Anatomy and Cell Biology, Saskatoon, Saskatchewan, Canada and* [2]*University of California, Department of Food Sciences and Technology, Davis, California, USA*

**ABSTRACT The pectoralis muscle of birds provides virtually all the power for the downstroke of the wing during flight. In adults it consists almost entirely of FOG (fast-twitch oxidative-glycolytic) and/or FG (fast-twitch glycolytic) fiber types. The aims of this study are to contrast MyHC (myosin heavy chain) transitions occurring within avian FG and FOG fibers during development, and to test the hypothesis that the pectoralis matures before the acquisition of flight. Pectoralis was obtained from pigeons (*Columba livia*) aged from 13 days *in ovo* to adult. Monoclonal antibodies generated against chicken MyHC isoforms were used with Western blots and immunocytochemistry. FG and FOG fibers were differentiated using a histochemical method demonstrating NADH (nicotinamide adenine dinucleotide), and "lesser fiber diameters" were quantified. Western blots confirm that the antibodies label pigeon MyHCs. A small number of the fibers are slow type *in ovo*, but these are quickly restricted in distribution and lost after hatching. *In ovo* fast-twitch fibers contain a ventricular isoform, and at least two embryonic-neonatal forms (designated E-N103 and E-N165). One week after hatching, fast-twitch fibers can be distinguished by NADH as FG or FOG. At fledging, four weeks after hatching, FG and FOG fibers are smaller than in older birds and E-N103 and E-N165 persist in both fiber types. E-N103 wanes in all fibers shortly after fledging. E-N165 gradually disappears from FG fibers. Thus, despite pigeons being at adult body mass at fledging, their pectoralis is not fully mature.**

KEY WORDS: *development, myosin, muscle, pigeon, flight*

## Introduction

The avian pectoralis muscle is an exemplary vertebrate locomotory muscle. Flapping (or active) flight is one of the most efficient forms of locomotion for distance traveled per unit of energy expended, but also one of the more costly in energy expended per unit of time (Norberg, 1990). On each side of the body, the pectoralis extends from the sternum and ribs to the humerus (Raikow, 1985). This muscle alone provides virtually all the power for the downstroke (powerstroke) of the wing during flight, and assists in decelerating the wing during its upstroke (Dial, 1992). Indicative of its importance, left and right pectoralis muscles together comprise from seven to 27% of the fresh body mass of a bird (Hartman, 1961).

The pectoralis consists almost exclusively (99 to 100%) of fast-twitch fiber types, in those birds relying upon flapping-flight (Rosser and George 1986; Rosser *et al.* 1996). Avian fast-twitch fibers are usually described as either fast-twitch oxidative-glycolytic (FOG) or fast-twitch glycolytic (FG) types. These fiber types differ in the activities of their energy-generating enzymes (Rosser and George, 1986; Torrella *et al.*, 1996), and in their constituent myosin heavy chain (MyHC) isoforms (Rosser *et al.*, 1996). Both energy-generating enzymes and MyHC isoforms have been directly correlated with the contractile properties of individual muscle fibers (Nemeth, 1990; Bottinelli *et al.*, 1994; Reiser *et al.*, 1996). FOG fibers are capable of sustained rapid contraction, and FG fibers of a more powerful but fast-fatiguing contraction.

*Abbreviations used in this paper:* MyHC, myosin heavy chain; FG, fast-twitch glycolytic; FOG, fast-twitch oxidative-glycolytic; NADH, nicotinamide adenine dinucliotide; HV11, 2E9, AB8, AG6, NA4, NA8, B103, EB165, monoclonal antibodies, generated in mouse against chicken myosin heavy chains; E-N103, myosin heavy chain (s) labeled by B103; E-N165, myosin heavy chain (s) labeled by EB165.

***Address for reprints:** University of Saskatchewan, College of Medicine, Department of Anatomy and Cell Biology, 107 Wiggins Road, Saskatoon, Saskatchewan, S7N 5E5, Canada. FAX: 1-(306)-966-4298. e-mail: Rosserb@duke.usask.ca

**#Present Address:** Ohio State University, Department of Animal Sciences, Columbus, Ohio, 43210, USA.

0214-6282/98/$10.00

Printed in Spain

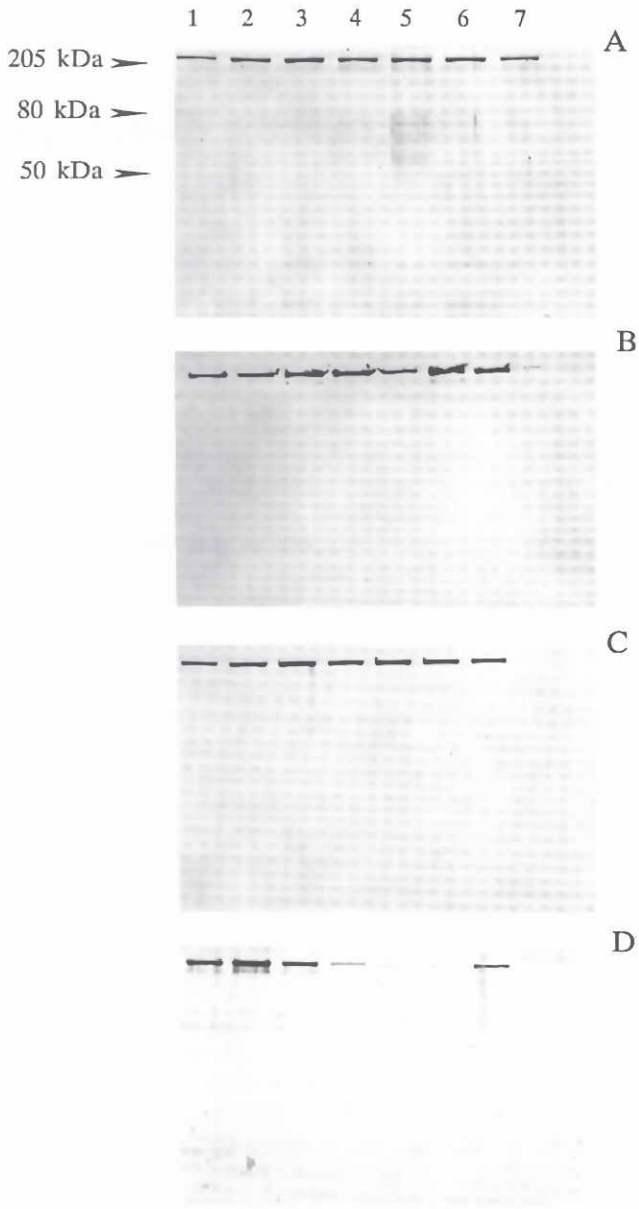


**Fig. 1. Western blot analysis of developing pigeon pectoralis myosin.** **(A)** *Myosin extracts from the pectoralis muscle of 1 week (lane 1), 2 week (lane 2), 3 week (lane 3), 6 week (lane 4), 12 week (lane 5), adult (lane 6) and 4 week (lane 7) pigeons analyzed on 10% SDS-PAGE and stained with Coomasie Blue R250. Duplicate gels were electrophoretically transferred to nitrocellulose and incubated with monoclonal antibodies AG6* **(B)**, *EB165* **(C)** *or B103* **(D)**. *The positions of the 205 kDa, 80 kDa and 50 kDa molecular weight standards are indicated by the arrows. Both AG6 and EB165 react strongly with myosin heavy chain in all the samples. B103, however, reacts most strongly with the pectoralis of the young (1-4 weeks), but declines during later developmental stages.*

MyHC transformations occurring during the development of FG fibers in the chicken and its close allies have been well documented (Merrifield *et al.*, 1989; Maruyama *et al.*, 1993; Bandman *et al.*, 1994). In chicken pectoralis muscle, which consists almost entirely of FG fibers, embryonic MyHC isoforms are supplanted after hatching by a neonatal isoform that is in turn replaced by an adult isoform (Bandman *et al.*, 1990). In muscles of the chicken other than the pectoralis, there can be different scenarios in which either embryonic or neonatal MyHC isoforms may predominate in mature FG fibers (Bandman *et al.*, 1994). However, to date developmental transitions occurring within avian FOG fibers have not been discerned.

While studies of mammalian muscle fibers have demonstrated precocial development of those muscles performing vital functions upon their initial usage, such as the diaphragm, the fibers in even these muscles undergo subsequent maturation and differentiation as the young grow (Kelly *et al.*, 1991; Finkelstein *et al.*, 1992; Pette and Staron, 1997). Success at fledging is crucial for survival, especially for altricial and semi-altricial birds (Gill, 1995; Stempniewicz, 1995). The young of most avian species, however, are essentially at their adult body mass before their first flight (Weathers, 1992; Gill, 1995). Body mass is strongly correlated with a number of parameters associated with avian flight (Rayner, 1988), including fiber type (Norberg, 1990). Therefore, one might expect a nearly mature phenotype in the pectoralis of newly fledged birds.

The purposes of this study are to contrast the MyHC transformations occurring during the development of avian FG and FOG fibers, and to test the hypothesis that these fibers in the pectoralis reach a mature state in their MyHC content before the acquisition of flight. The experimental model utilized is the pectoralis muscle of the domestic pigeon *(Columba livia)* which, in the adult, comprises 20% of the total body mass (Hartman, 1961) and consists of well defined populations of FG and FOG fibers (George and Berger, 1966; Torrella *et al.*, 1993; Tobalske *et al.*, 1997). Young pigeons are altricial, flightless, and confined to the nest until about four weeks of age (Vriends, 1988). They reach their adult body mass before fledging (Abs, 1983). We employ monoclonal antibodies originally generated against known chicken MyHC isoforms, to study muscles from pigeons aged from thirteen days *in ovo* through to adult. We find that while FG and FOG fibers appear to contain similar MyHC isoforms in the earlier stages of development, their MyHC content diverges after hatching. We also demonstrate that the muscle as a whole is not fully mature at fledging.

## Results

### *Western blot analysis*

Results (Fig. 1) show that both AG6 (Fig. 1B) and EB165 (Fig. 1C) react strongly with myosin heavy chain purified from the pectoralis of post-hatch pigeons. B103 (Fig. 1D), however, reacts most strongly with the pectoralis of the young birds (1-4 weeks), and wanes during subsequent developmental stages.

### *Histochemistry*

*In ovo* (not shown) and at one day after hatching (Fig. 3A), on the basis of staining for NADH activity we were unable to differentiate fibers in the pectoralis as either FG or FOG. We could, however, distinguish FG or FOG fibers on this basis in all subsequent stages: one week (Fig. 4A), two weeks (Fig. 5A), three weeks (Fig. 6A), four weeks (Fig. 6D), four and one-half weeks (Fig. 6G), six weeks (Fig. 7A), 12 weeks (Fig. 7D) and adult (Fig. 7G).

### *Immunocytochemistry*

Labeling of pigeon pectoralis by the monoclonal antibodies used in this study is summarized in Table 1. The specificity of each of these antibodies against chicken MyHCs, and the MyHCs of

other birds, has been documented elsewhere (Rosser *et al.*, 1996). Due to minor variations among muscles in experimental protocol and darkroom procedure, the intensity of the labeling among different pigeons ought not be considered for subtle quantitative comparisons.

NA4 which labels all avian MyHCs studied to date (see Rosser *et al.*, 1996), labels all fibers in the present study (Table 1). Similarly AG6, which reacts with almost all avian fast-twitch fibers, labels all fibers here with the exception of NA8 positive fibers (Table 1).

EB165 labels a variety of avian fast-twitch MyHCs, including both embryonic and adult MyHCs in the fast-twitch fibers of the chicken pectoralis (Rosser *et al.*, 1996). This antibody can also differentiate FG and FOG fibers in the pectoralis of the mature pigeon (see Rosser *et al.*, 1996); while FOG fibers are labeled, FG fibers are not. In the present study, EB165 labels all fast-twitch fibers *in ovo* (Table 1; Fig. 2A) and in pigeons aged from one day through to two weeks after hatching (Figs. 3B,4B and 5B). However, EB165 labeling wanes in the FG fibers of birds aged from three to six weeks (Figs. 6B,E,H and 7B). In all older birds, EB165 labeling is wholly absent in FG fibers but undiminished in FOG fibers (Fig. 7E and H).

B103 labels both embryonic and neonatal MyHCs in the fast-twitch fibers of the chicken pectoralis, but is fairly limited in its reactivity with mature muscle from most species (Rosser *et al.*, 1996). Present in all fast-twitch fibers of the pectoralis of pigeons *in ovo* (Table 1) and aged from one day through to three weeks after hatching (Figs. 3C,4C,5C and 6C), B103 labeling progressively diminishes throughout the muscle from three to four and one-half weeks (Fig. 6C,F and I). It does, however, disappear less rapidly from the FG fibers (Fig. 6I). B103 labels only the smallest diameter fibers in birds six weeks (Fig. 7C) and older (not shown).

2E9 and AB8, respectively, label neonatal and adult MyHCs in fast-twitch fibers of the chicken pectoralis (Bandman *et al.*, 1990). However, work to date has indicated that labeling by these antibodies is restricted to chickens and their close allies within the order galliformes (Rosser *et al.*, 1996). In the present study, they do not label pigeon pectoralis of any age (not shown).

HV11 normally reacts with ventricular MyHC, and with certain embryonic and regenerating chicken skeletal muscle fibers (Hartley *et al.*, 1991). Its reactivity with mature avian fibers is extraordinarily rare (Rosser *et al.*, 1996). In the present study, it labels most fast-twitch fibers at 13 days *in ovo* (Table 1). Subsequently, labeling decreases so that by hatching only a tiny minority of the fibers throughout the muscle react with this antibody (Fig. 3E). By one week of age HV11 labels even fewer fibers, which are comparatively minute in diameter (Fig. 4E). HV11 does not react with any fibers in the pectoralis two weeks or older (not shown).

NA8 labels slow myosin in avian muscle (Rosser *et al.*, 1996). In pigeon pectoralis, it labels only a small minority of fibers in the youngest birds (Table 1; Fig. 2B and 3F). Although NA8 positive fibers are distributed throughout the muscle at 13 days *in ovo*, they become restricted in distribution within the muscle as the birds age. By one week of age, labeling by NA8 was absent throughout the bulk of the muscle (Fig. 4F). However, until three weeks of age, they persisted as a tiny proportion of the fibers localized along one edge of the muscle (not shown). These NA8 positive fibers were negative for either EB165 or B103 labeling. In post-hatch birds, they were NA4 positive but AG6 negative (Table 1). By four weeks of age, despite a fairly extensive search of both superficial and deep areas of the muscle, we were unable to locate any fibers labeled by NA8.

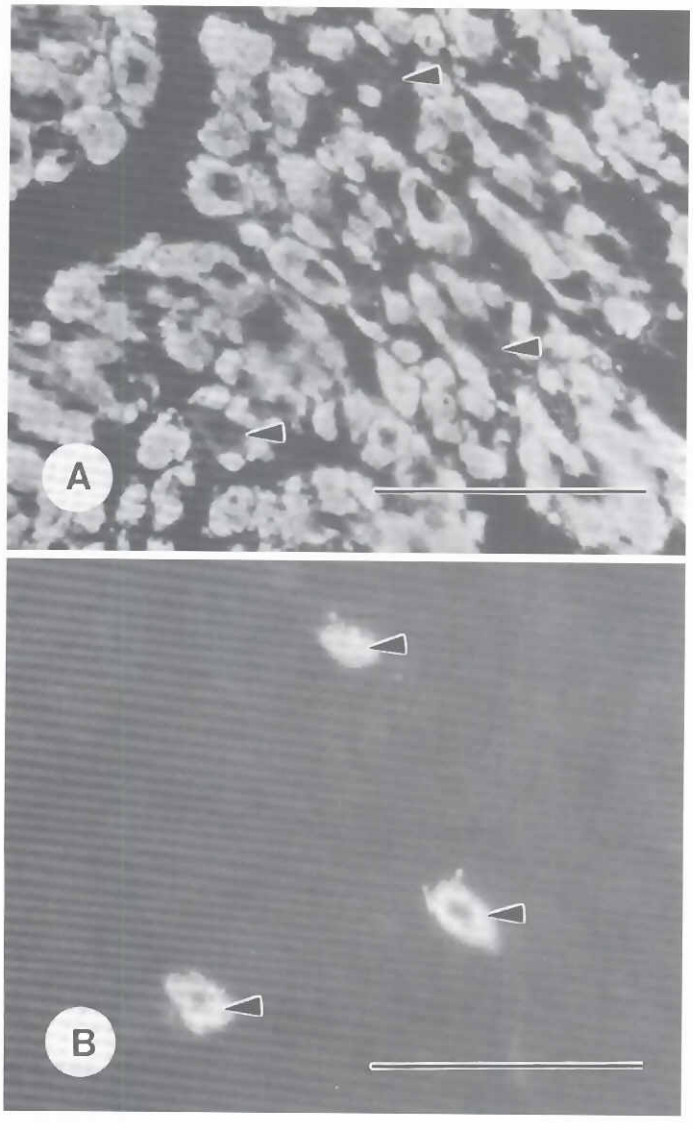


**Fig. 2. Transverse section of the pectoralis muscle from a pigeon 16 days *in ovo*.** *Section double-labeled by* **(A)** *fluorescein-conjugated EB165 and* **(B)** *NA8. Arrowheads indicate that the same three fibers not labeled by EB165 are labeled by NA8. These three fibers are slow fibers. Bar, 50 μm.*

### *Quantitative measurements*

The mean body mass of the pigeons at each representative post-hatch age is shown in Figure 8. The birds reach their adult mass by three weeks of age. The measurements of 'lesser fiber diameter' reveal that neither FOG nor FG fibers reach their adult proportions before the acquisition of flight (Fig. 9). FOG fibers are adult size by six weeks of age. FG fibers, however, do not reach their mature size until after six weeks of age.

## Discussion

### *Slow fibers*

Slow fibers are numerous within the pectoralis of the Ostrich and other ratites (Rosser *et al.*, 1996). Certain species that have mastered soaring flight, such as vultures and pelicans, have an accessory deep belly of the muscle consisting entirely of slow

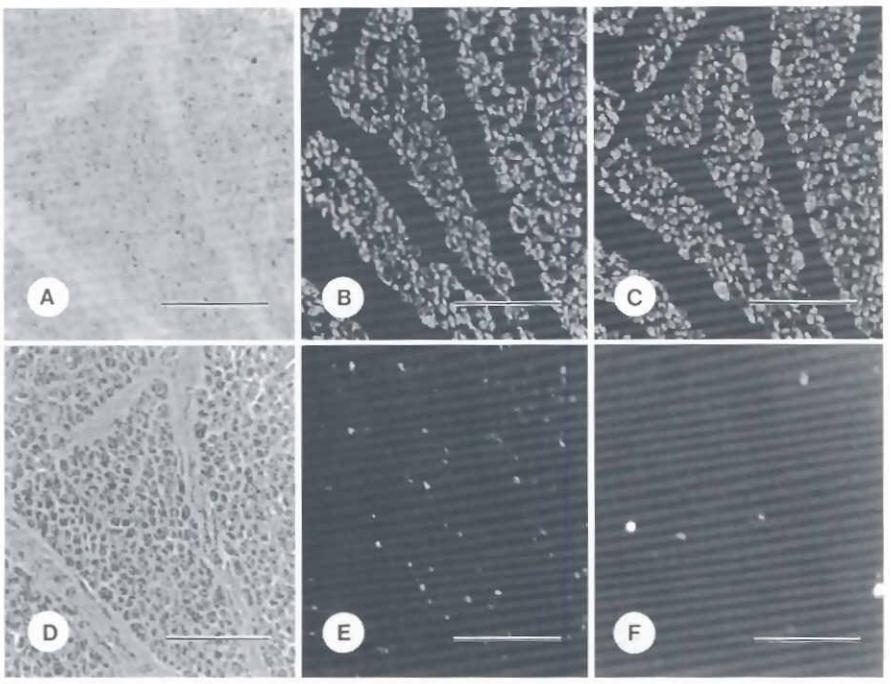


**Fig. 3. Transverse serial sections of the pectoralis muscle from a pigeon one day after hatching. (A)** *Section stained for NADH activity,* **(B)** *section labeled by EB165,* **(C)** *section labeled by B103,* **(D)** *phase contrast of section shown in (A),* **(E)** *section labeled by HV11 and* **(F)** *section labeled by NA8. At this early stage of development, FOG and FG fibers cannot be differentiated. A minority of the fibers are labeled by HV11, and an even smaller proportion by NA8. Bar, 50 μm.*

fibers (Meyers and Mathias, 1997). If present in the mature pectoralis of any other species, however, slow fibers are restricted to a tiny deep area termed the 'red strip' (Rosser and George, 1986). Although present throughout the pectoralis in the early chick embryo, at hatching these slow fibers are restricted to the deeper regions where they are further localized during subsequent maturation (Matsuda *et al.*, 1983).

A minor population of slow fibers is present within the pigeon pectoralis up to three weeks after hatching. They seem comparable in their numbers and development to the slow fibers in the chicken 'red strip'. We did not, however, locate any slow fibers within the pectoralis of pigeons older than three weeks posthatch.

### *How many fast-twitch isoforms in pigeon pectoralis?*

Labeling by each of the monoclonals HV11, B103 and EB165, terminated at different times during development. Although HV11 labeled the smallest diameter fibers by one week after hatching, it did not identify any fibers by two weeks. B103 labeling all but disappeared from both FOG and FG fiber types just after fledging at four weeks: it did, however, still label the tiniest fibers. EB165

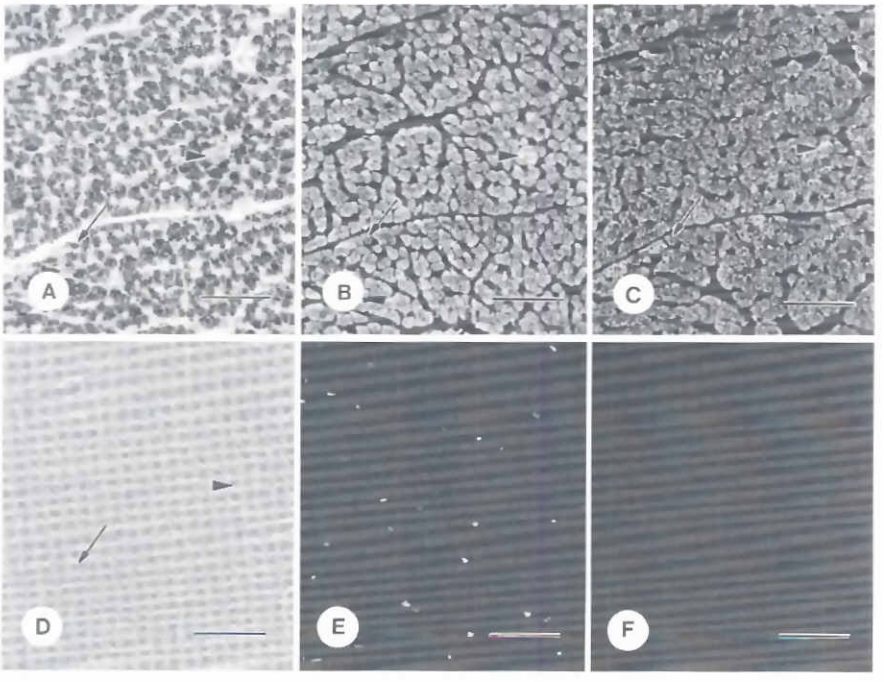


**Fig. 4. Transverse serial sections of the pectoralis muscle from a pigeon one week after hatching.** *Large arrowhead indicates a FG fiber, and small arrow a FOG fiber.* **(A)** *Section stained for NADH activity,* **(B)** *section labeled by EB165,* **(C)** *section labeled by B103,* **(D)** *phase contrast of section shown in (E),* **(E)** *section labeled by HV11 and* **(F)** *section labeled by NA8. The same FG and FOG fibers are followed in sections (A) through (D). At this stage of their development, FOG and FG fibers can be differentiated by their size and NADH activity; characteristically, FG fibers have a larger 'lesser fiber diameter' and are more lightly stained for NADH activity than are FOG fibers. Neither EB165 nor B103 antibodies differentiate fiber types. HV11 still reacts with a tiny minority of the fibers, which are comparatively minute in diameter. NA8 labeling is absent from this section. Bar, 50 μm.*

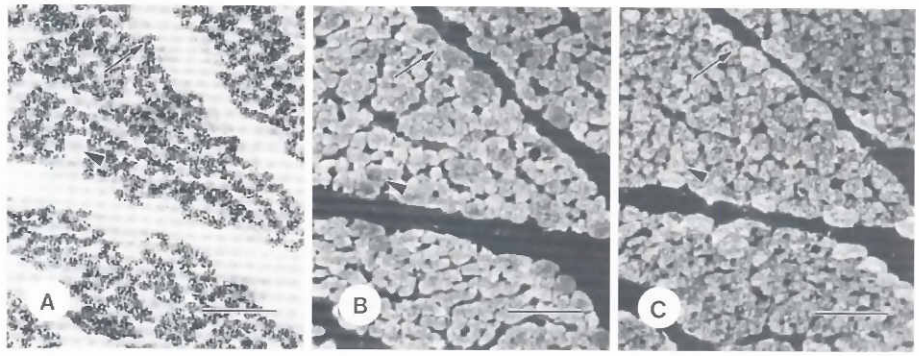


**Fig. 5. Transverse serial sections of the pectoralis muscle from a pigeon two weeks after hatching.** *Large arrowhead indicates a FG fiber, and small arrow a FOG fiber.* **(A)** *Section stained for NADH activity,* **(B)** *section labeled by EB165,* **(C)** *section labeled by B103. The same FG and FOG fibers are followed in (A) through (C). As observed in the one week old birds (Fig. 4), FG and FOG fibers are differentiated on the basis of their NADH activity only. However, by this stage of development neither NA8 nor HV11 antibodies label the fibers (not shown). Bar, 50 μm.*

labeling was retained in the FOG fibers, but vanished from the FG fibers after six weeks posthatch. This indicates that HV11, B103 and EB165 were each labeling a different epitope. Furthermore, when their labeling waned, each of those epitopes must have been on a different isoform. If these deductions are inaccurate, labeling by two or more of these antibodies would have ceased simultaneously.

Our results mark at least four isoforms: HV11 positive, B103 positive, EB165 positive and 'Negative' (labeled by NA4 and AG6, but not labeled by HV11, B103 or EB165). Each of these, however, may be more than one isoform. It would appear that, as in the chicken, a ventricular isoform labeled by HV11 characterizes early embryonic fibers in the pigeon pectoralis. An embryonic-neonatal isoform, which we shall term E-N103, is characterized by B103 in the pigeon pectoralis. Another embryonic-neonatal form, which we term E-N165, is characterized by EB165. Like an embryonic isoform in the chicken pectoralis, both E-N103 and E-N165 are present in the earliest stages of the pigeon pectoralis. Like the neonatal isoform in the chicken, both persist in the pigeon muscle well after hatching. The 'Negative' isoform, like the adult isoform in the chicken pectoralis, does not appear to be expressed in any appreciable concentration in the pigeon pectoralis until well after hatching.

### *Myosin in fiber ends*

Neonatal isoform is retained in the terminal tips (ends) of the muscle fibers in the mature chicken pectoralis (Rosser *et al.*, 1995). Characteristically, the smallest diameter fibers seen in cross-sections are in reality the tips of larger diameter fibers (Rosser *et al.*, 1995). In the present study, HV11 labeled the tiniest fibers up to one week after hatching and B103 the tiniest fibers in mature muscle. These results imply that one week after hatching the HV11 positive isoform is retained at the fiber ends, and that the E-N103 isoform remains at the ends of mature fibers.

### *Different MyHC transformations in FOG and FG fibers*

Each mammalian mature fast-twitch fiber type is characterized by a unique MyHC isoform. During development, each of these fast-twitch fiber types typically arises from fibers which first express an embryonic and then a neonatal isoform (Schiaffino and Reggiani *et al.*, 1994; Pette and Staron, 1997). In birds, each mature fast-twitch fiber type also appears to have a distinct MyHC isoform (Rosser *et al.*, 1996). In mature avian muscles, however, it is more common for fast-twitch fibers to retain either their embryonic or neonatal isoform as the mature isoform (Bandman *et al.*, 1994).

Our results indicate that while FG and FOG fibers in the pigeon pectoralis share several of the same MyHC developmental isoforms, they do ultimately follow different developmental paths. The very early HV11 isoform almost totally disappears from both fiber types *in ovo*. E-N103 and E-N165 are present from the earliest stages. However, E-N103 begins to decrease by three weeks after hatching and is finally lost from both fiber types just after the birds fledge. E-N165, by comparison, is retained in the FOG fibers but totally lost from the FG fibers by six to twelve weeks after hatching. The

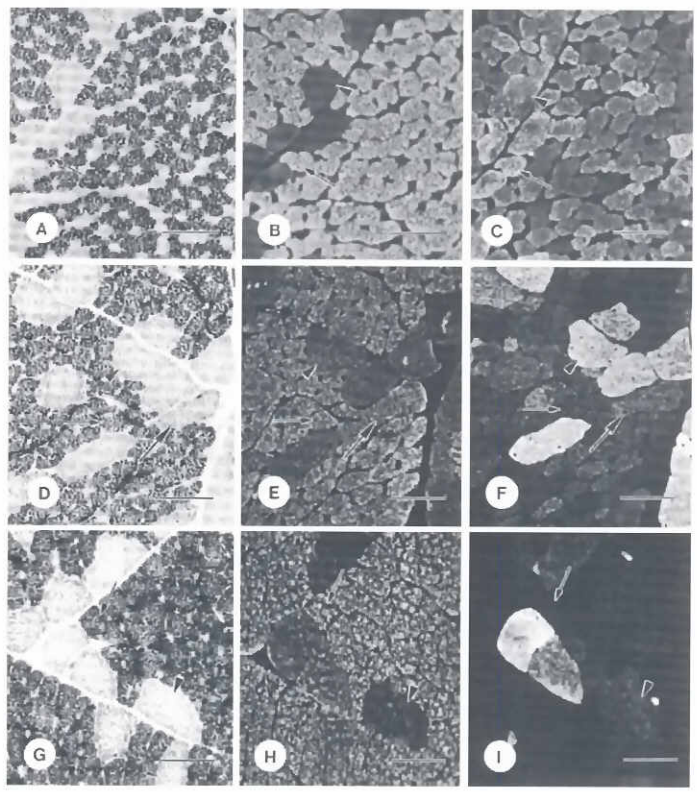


**Fig. 6. Transverse serial sections of the pectoralis muscle from pigeons aged three, four and four and one-half weeks after hatching.** *Large arrowhead or larger arrow indicate a FG fiber, and smaller arrow a FOG fiber. Serial sections are from the pectoralis of birds three weeks* **(A, B** *and* **C)**, *four weeks* **(D, E** *and* **F)** *and four and one-half weeks* **(G, H** *and* **I)** *after hatching. Sections were stained for their NADH activity (A, D and G), labeled by EB165 (B, E and H) or B103 (C, F and I). The same FG and FOG fibers are followed in (A) through (C), (D) through (F) and (G) through (I). During these crucial one and one half weeks of development, as the birds fly after four weeks of age, FOG and FG fibers continue to be differentiated on the basis of their NADH activity. EB165 labeling begins to wane in some of the FG fibers. B103 labeling starts to subside in both FOG and FG fibers, albeit more rapidly in the former than later. Bar, 50 μm.*

absence of labeling of the mature FG fibers by three of our antibodies (NA4 and AG6 positive, but HV11, B103 and EB165 negative) is indicative of the presence of another isoform (which we designate as the 'Negative' isoform). Presumably, as other isoforms wane within the FG fibers the 'Negative' isoform increases.

### *Pectoralis not fully mature at fledging*

Young pigeons are altricial, flightless and confined to the nest until about four weeks of age (Levi, 1963), after which they start to spread their wings and practice flying in the loft (Vriends, 1988). At this time they can be vulnerable to attack by older birds (Vriends, 1988) until they are independent by 30 to 35 days (Levi, 1963).

The four week post-hatch birds in the present study had been making preliminary sorties within the loft for approximately one day. The bird that was four and one-half weeks post-hatch, and all older birds, were completely independent and flying outside the loft. Regardless, fiber diameters reveal that at fledging neither FOG nor FG fiber types had reached their adult dimensions. The immaturity of the fiber types is further corroborated in that they still contain the E-N103 isoform. Thus, our initial hypothesis that muscle fibers in the pigeon pectoralis muscle reach their mature state before the acquisition of flight is "not" verified.

TABLE 1

**MONOCLONAL ANTIBODY BINDING TO FIBER TYPES FOUND IN THE PECTORALIS MUSCLE OF THE DOMESTIC PIGEON DURING REPRESENTATIVE DEVELOPMENTAL AGES**

| | | Monoclonal Antibody[1] | | | | | |
|---|---|---|---|---|---|---|---|
| **Age[2]** | **Fiber Type[3,4]** | **NA4** | **AG6** | **EB165** | **B103** | **HV11** | **NA8** |
| 13 day | slow | | | - | - | | + |
| *in ovo* | fast | + | + | + | + | ± | - |
| 16 day | slow | | | - | - | | + |
| *in ovo* | fast | + | + | + | + | ± | - |
| 1 day | slow | + | - | - | - | | + |
| | fast | + | + | + | + | ± | - |
| 1 week | slow | + | - | - | - | | + |
| | FG | + | + | + | + | ± | - |
| | FOG | + | + | + | + | ± | - |
| 2 week | slow | + | - | - | - | - | + |
| | FG | + | + | + | + | - | - |
| | FOG | + | + | + | + | - | - |
| 3 week | slow | + | - | - | - | - | + |
| | FG | + | + | ± | ± | - | - |
| | FOG | + | + | + | ± | - | - |
| 4 week | FG | + | + | ± | ± | - | - |
| | FOG | + | + | + | ± | - | - |
| 6 week | FG | + | + | ± | - | - | - |
| | FOG | + | + | + | - | - | - |
| 12 week | FG | + | + | - | - | - | - |
| | FOG | + | + | + | - | - | - |
| Adult | FG | + | + | - | - | - | - |
| | FOG | + | + | + | - | - | - |

1 Immunofluorescence: + = positive reaction, - = negative reaction, ± =variable reaction among fibers, a blank space indicates not done

2 unless indicated as *in ovo*, refers to age after hatching

3 fast/slow indicates that the overwhelming majority of the fibers were fast-twitch; only the tiny minority labeled by NA8 could be considered slow. At the earliest stages of development, neither metabolic criteria nor antibodies against (MyHCs) could distinguish fast fibers as FG or FOG

4 fast fibers were classified to type by their nicotinamide adenine dinucleotide (reduced form) activity as either FG (fast-twitch glycolytic) or FOG (fast-twitch oxidative-glycolytic)

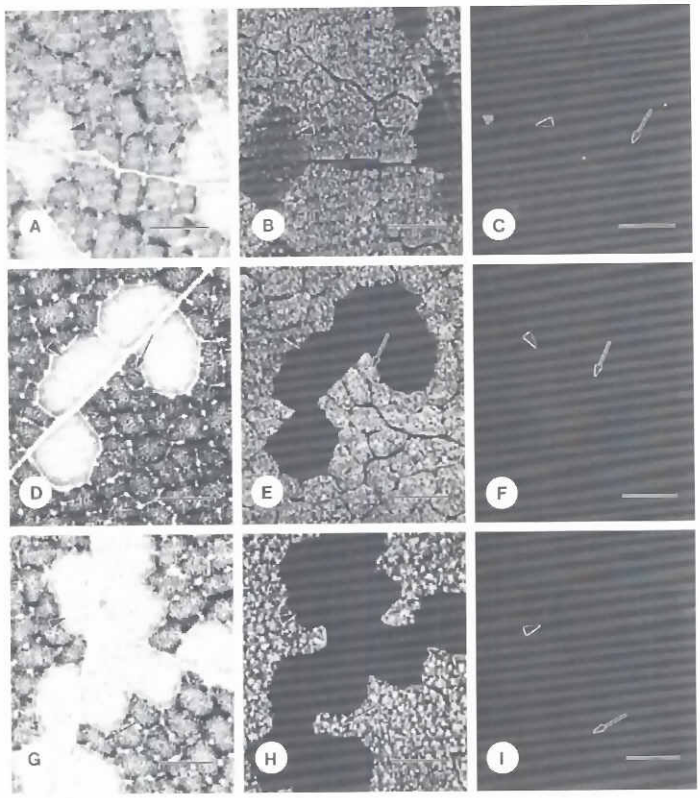


**Fig. 7. Transverse serial sections of the pectoralis muscle from pigeons aged six weeks, twelve weeks and at least one year after hatching.** *Large arrowhead indicates a FG fiber, and smaller arrow a FOG fiber. Serial sections are from the pectoralis of birds six weeks* **(A, B** *and* **C)**, *twelve weeks* **(D, E** *and* **F)** *and at least one year* **(G, H** *and* **I)** *after hatching. Sections were stained for their NADH activity (A, D and G), labeled by EB165 (B, E and H) or B103 (C, F and I). The same FG and FOG fibers are followed in (A) through (C), (D) through (F) and (G) through (I). During this maturation of the muscle, FG and FOG fibers continue to be clearly differentiated by their NADH activity. While EB165 labeling strongly persists in all FOG fibers, it further declines and then disappears from all FG fibers. B103 labeling is almost absent from these later stages in both FG and FOG fibers, appearing only in a minority of fibers which are comparatively minute in diameter (see Fig. 7C). Bar, 50 μm.*

### *Some functional considerations*

While differentiation of muscle fibers in the rat diaphragm precedes that of the limb muscles, there is still a great deal of fiber differentiation and maturation within the diaphragm after birth (Kelly *et al.*, 1991; Pette and Staron, 1997). Similarly, although muscle fibers within the limbs of newborn sheep are as precocial and crucial for survival as are their diaphragm fibers, sheep limb and diaphragm muscle fibers alike undergo additional maturation and differentiation after birth (Finkelstein *et al.*, 1992). In each of these examples, the subsequent maturation of the muscles is associated with growth of the young and modifications of muscle function(s).

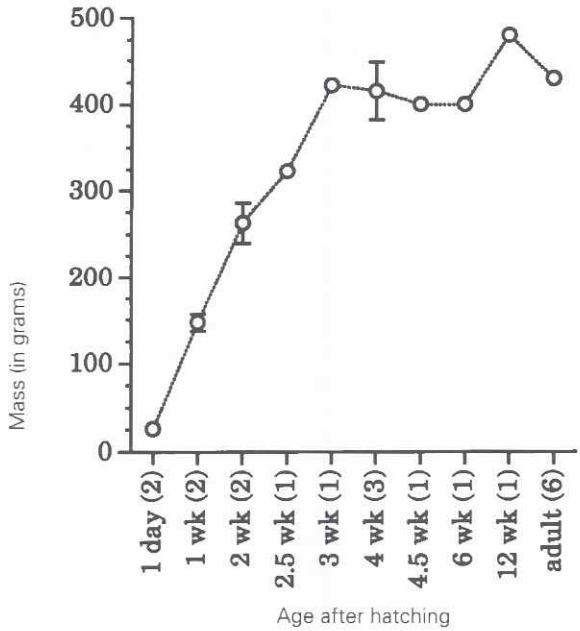


**Fig. 8. Body mass (in grams) of pigeons during development.** *Number in brackets after each age indicates the number of birds studied. Data is expressed as Mean ±SE (standard error). In certain instances, such as the one day old birds and the adults, the standard error was too close to the mean to be expressed graphically. These pigeons reach their mature body mass by three to four weeks of age, prior to the acquisition of flight after four weeks.*

The greatest force output required from the pigeon pectoralis muscle is during takeoff and vertical ascending flight (Dial and Biewener, 1993). It is thought that FG fibers are employed during takeoff, but derecruited during level flight (Welsford *et al.*, 1991). In the present study, the more gradual maturation of the FG fibers attests to a gradual acquisition of a more powerful flight performance.

We did not quantify flight performance of newly fledged pigeons, nor can we reference any. However, pigeon fanciers do not recommend training birds for racing until the birds are aged from six to twelve weeks after hatching (Levi, 1963), and the birds appear to be weak fliers until that time (Ken King, personal communication). This is the age at which our data indicate that the pectoralis reaches its mature profile.

## Materials and Methods

### *Pigeons and tissue preparation*

Domestic pigeons (*Columba livia*) bred for racing were obtained during August and September of 1992. Pigeons ranging in age from thirteen days *in ovo* to adult were housed in an insulated, shielded, loft located outdoors in Saskatoon (52°10'N106°40'W) Canada. While the four week old birds had just begun to make weak sorties within the loft, all birds older than four weeks were independent and flying regularly outside the loft. The birds were collected by early fall, while the climate was temperate. Each bird was killed by an overdose of sodium pentobarbital injected into its abdominal cavity.

Blocks of muscle were excised from the more superficial areas of either the right or left pectoralis muscle of each bird, as in our earlier work (Rosser *et al.*, 1996). These areas of the muscle are known to consist entirely of fast-twitch fibers in mature birds (Rosser *et al.*, 1996). In the younger birds, a block could contain most of the muscle. Blocks were coated with Tissue-Tek O.C.T. Compound (Miles Inc., Elkhart, Indiana), and then quick frozen in 2-methylbutane cooled to -160°C by liquid nitrogen (Dubowitz, 1985). They were stored at -80°C until sectioned.

Sections of 4 to 6 microns thickness were cut from blocks representative of each bird, in a cryostat maintained at -20°C. Two to three sections were picked up on each microscope slide, and an extensive series of serial sections was obtained from each muscle.

### *Fiber typing*

Avian fast-twitch and slow fibers are commonly distinguished by either their myosin ATPase activity (Rosser and George, 1986; Torrella *et al.*, 1993) or their reactivity to antibodies against specific epitopes located on various MyHC isoforms (see Rosser *et al.*, 1996). Avian fast-twitch fibers are then usually classified as either fast-twitch glycolytic (FG) or fast-twitch oxidative-glycolytic (FOG) types based on the biochemical pathways used to produce the energy for contraction (Rosser and George 1986; Rosser *et al.*, 1996). However, different nomenclatures do certainly exist (see Torrella *et al.*, 1993; Tobalske *et al.*, 1997). Histochemical stains for mitochondrial oxidative enzymes are routinely used for this purpose. Smaller diameter fibers stain dark and larger fibers light, as there is an inverse correlation between fiber size and mitochondrial density (George and Berger, 1966). These fibers are then classified, respectively, as FOG and FG. While avian pectoralis can contain 'intermediate' fibers between FG and FOG in their energy-generating enzyme activities, fibers in the pigeon pectoralis are clearly either FG or FOG (George and Berger, 1966).

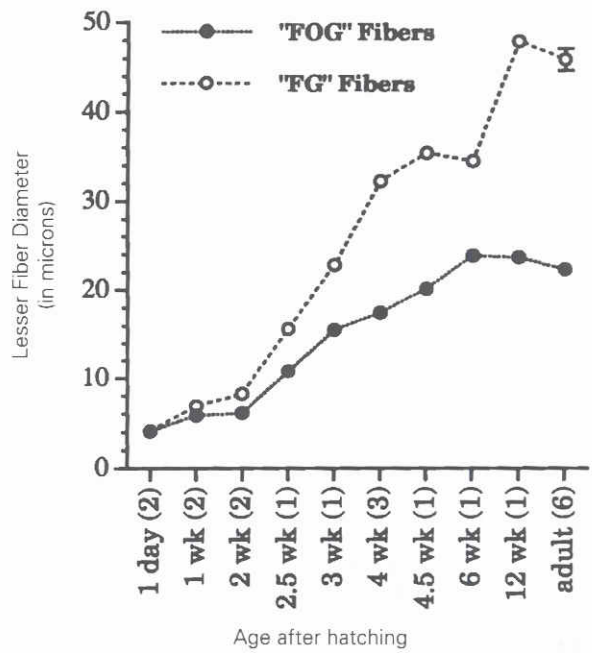


**Fig. 9. 'Lesser fiber diameter' (in microns) of type FG and type FOG fibers during development of the pigeon pectoralis muscle.** *Number in brackets after each age indicates the number of birds studied. Data is expressed as Mean ± SE (standard error). In all but one instance, the adult FG fibers, the standard error was too close to the mean to be expressed graphically. Neither fiber type reaches its adult proportions prior to the acquisition of flight, four and one half weeks after hatching. FOG fibers are adult size by six weeks of age. FG fibers, however, do not reach their mature size until after six weeks of age.*

***Histochemistry***

One slide from each series of sections was stained by histochemical methods (Dubowitz, 1985) for the demonstration of NADH (nicotinamide adenine dinucleotide, reduced form). NADH is a product of the citric acid cycle and fatty acid oxidation (Voet and Voet, 1995). It is prevalent in mitochondria (Dubowitz, 1985), and used to distinguish FOG from FG fibers (Rosser *et al.*, 1996).

***Myosin heavy chain isoforms and monoclonal antibodies***

At least nine myosin heavy chain (MyHC) isoforms occur in chicken muscles (see Bandman *et al.*, 1990,1994). These isoforms are differentiated by a library of monoclonal antibodies generated against them: NA4, AG6, EB165, AB8, 2E9, B103, HV11 and NA8 (see Bandman *et al.* 1990,1994). The specificity of these antibodies against avian myosin heavy chains (MyHCs), using immunoblots and immunocytochemistry, have been detailed elsewhere (Bandman, 1985; Cerny and Bandman, 1987; Bandman and Bennett, 1988; Bourke *et al.*, 1991). Their reactivity against known chicken MyHCs has been summarized elsewhere (Rosser *et al.*, 1996).

***Epitopes and isoforms***

Each of the monoclonal antibodies used recognizes just one small epitope (or amino acid sequence) on a MyHC isoform (Moore *et al.*, 1992). However, the same epitope may occur on several different MyHC isoforms. Consequently, the antibody against that epitope will label each isoform containing the epitope.

***Immunocytochemical techniques***

Immunocytochemical methods used have been previously detailed (see Rosser *at al.*, 1996). Primary antibodies NA4, AG6, EB165, AB8, HV11 and NA8 were each used at a dilution of 1:5,000, and 2E9 and B103 at 1:2,000. While these antibodies have very high affinities for MyHC isoforms, different dilutions are used to compensate for minor differences in binding affinities (Bandman, 1985; Cerny and Bandman, 1987; Bandman and Bennett, 1988; Bourke *et al.*, 1991). Each slide from each series received a different primary antibody. Binding of the primary antibodies was visualized by using a fluoresceinated horse anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, California) at a dilution 1:64.

Double immunofluorescent labeling was used to study the thirteen and sixteen day embryos, by modifying the protocol outlined in the preceding. After labeling slides with either B103 or NA8, binding was visualized by using anti-mouse IgG Rhodamine (TRITC) conjugate (Sigma Chemical Co., St. Louis, Missouri) diluted 1:64. The same sections were then treated with fluorescein-conjugated EB165 (according to Hartley *et al.*, 1991) diluted 1:1,000.

***Western blots***

SDS-PAGE and Western blot analyses determine whether the antibody reactivities shown by our immunocytochemical techniques were due to antibody affinity for MyHC(s). From post-hatch specimens, pectoralis muscle (approx. 0.5 $cm^3$) was excised from the frozen tissue blocks embedded in O.C.T. Compound. The O.C.T. was cut away at -20°C with a razor blade, and myosin was extracted (see Bandman *et al.* 1982). Myosin samples were analyzed by SDS-PAGE on 10% polyacrylamide gel, stained with Coomassie Blue R250 (0.0004% in 40% methanol and 5% acetic acid), and then electrophoretically transferred to several pieces of nitrocellulose (see Bandman, 1985). Nitrocellulose blots were then incubated with 5% nonfat milk powder, 3% Tween-20 in PBS (PBS-Tween Milk) for 30 min at 37°C. Subsequently, for 60 min at room temperature with agitation, each blot was incubated with one of the following monoclonal antibodies: AG6, EB165 or B103. Antibodies were diluted in PBS-TM: EB165 (1:25,000), AG6 (1:50,000) and B103 (1:10,000). Blots were then washed three times in PBS-Tween Milk over 15 min and, subsequently, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories, Pennsylvania; 1:1,000 in PBS-TM) for 60 min at room temperature with agitation. Blots were then washed three times in a 0.1 M Tris buffer, pH 7.4 over 15 min, and developed in 0.1 M Tris, pH 7.4 containing diaminobenzidine (0.2 mg/ml), $H_2O_2$ (0.024%).

***Quantitative measurements***

As body mass is correlated with a number of parameters associated with avian flight, each post-hatch bird was weighed immediately after death.

A Wild-Leitz Ortholux microscope, with an attached Hitachi videocamera connected to an FT-100 videoboard (Imaging Technology, Vancouver) mounted inside a DN-4000 graphics workstation (Apollo Computer, Calgary), was used to measure the "lesser fiber diameters" of representative populations of muscle fibers. 'Lesser fiber diameter' is used to overcome distortion which can occur if a fiber is cut obliquely rather than transversely. It is defined as the maximum diameter across the lesser aspect of a fiber (Dubowitz, 1985). At least 50 fibers of each type per post-hatch pigeon were measured. This enabled us to construct a profile of the changing sizes of the FOG and FG fibers during development.

*Acknowledgments*

*We extend our deepest appreciation to Mr. Ken King, of Saskatoon, who was generous enough to provide from his aviary all pigeons used in this study. In addition, we thank Mr. King for taking the time to share some of his in-depth knowledge of these birds. Access to the DN-4000 graphics workstation was cordially granted by Dr. Scott Lozanoff, formerly of the Department of Anatomy and Cell Biology, University of Saskatchewan, and currently of the Department of Anatomy, University of Hawaii. This study was supported by operating grants awarded to B.W.C.R. by the Saskatchewan Health Research Board and by the Natural Sciences and Engineering Research Council of Canada.*

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*Received: December 1997*
*Accepted for publication: March 1998*