CHANGES IN MYOGLOBIN AND LACTATE DEHYDROGENASE IN MUSCLE TISSUES OF A DIVING BIRD, THE PIGEON GUILLEMOT (CEPPHUS COLUMBA), DURING MATURATION

by

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Physiological adaptations of heart and pectoralis muscle tissues to diving-induced hypoxia were compared among three stages of maturation of the Pigeon Guillemot (Cepphus columba); chick, fledgling, and adult. Myoglobin concentration increased from fledgling to adult heart and pectoralis muscles, while myoglobin polypeptide expression changed between chick and fledgling pectoralis muscles. Total lactate dehydrogenase (LDH) activities in pectoralis muscle increased from chick to fledgling. The ratio of LDH-5 to LDH-1, 2, 3, and 4 in fledgling pectoralis was greater than that in chick and adult pectoralis. Hematocrits and blood oxygen capacities increased from chick to fledgling to adult. Pigeon Guillemots, as they mature from chicks to adults, become better adapted to exercise and diving. Aerobic metabolism is preferred in adult heart and pectoralis muscles, although pectoralis muscles potentially resort to anaerobic metabolism during temporary periods of hypoxia.

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CHAPTER I

INTRODUCTION

The physiology of exercise and diving has been a topic of great interest to biologists (Scholander, 1940; Andersen, 1966; Ridgway and Scronce, 1969; Kooyman, 1972; Kerem et al., 1973; Hochachka et al., 1975; Jones, 1976; Butler and Jones, 1982; Mill and Baldwin, 1983; Deshpande et al., 1984; Castellini et al., 1985; Grigg et al., 1986; Hochachka, 1986). One main focus of these studies is how animals respond to lowered oxygen tension (hypoxia). Studies on a wide range of animals have shown numerous behavioral, physiological, and biochemical adaptations to hypoxia (Irving et al., 1941; Andersen, 1966; Vesell, 1966; Cohen, 1969; Ridgway and Scronce, 1969; Kooyman, 1972, 1985; Kerem et al., 1973; Hochachka and Storey, 1975; Butler and Jones, 1982; Castellini et al., 1985).

Diving induces hypoxia; adaptations to hypoxia include apnea, bradycardia, and peripheral vasoconstrictions--collectively known as the diving response (Scholander, 1940; Catlett and Johnston, 1974; Zapol et al., 1979; Murphy et al., 1980; Butler and Jones, 1982; Butler and Woakes, 1984; Hochachka, 1986). These adaptations insure that oxygen is reserved primarily for the organs most critical to sustaining life--the brain, heart, and lungs (Bron et al., 1966; Angell-James and Daly, 1969; Butler and Jones, 1971; Andersen and Blix, 1974; Jones et al., 1979).

At the molecular level, skeletal muscles are specially adapted to tolerate hypoxic conditions (Scholander et al., 1942; Simon et al., 1974; Hochachka and Storey, 1975; Castellini and Somero, 1981; Castellini et al., 1981; Hochachka, 1985). Myoglobin facilitates the diffusion of oxygen into cells (Wittenberg, 1959; Hemingsen, 1963; Kreuzer, 1970; Wittenberg et al., 1975; Cole, 1983), but may also act as an oxygen storage molecule in muscle tissue. A relatively high myoglobin concentration in muscle tissue is one adaptation to hypoxia. Diving animals have higher concentrations of myoglobin in their muscle tissue than non-diving animals. This oxygen store is available during periods of hypoxia (Theorell, 1934; Kendrew et al., 1954; Ridgway and Johnston, 1966; Lenfant, 1969; Blessing, 1972; Weber et al, 1974; Castellini and Somero, 1981; Mill and Baldwin, 1983).

Another mechanism of adaptation is the enhancement of glycolytic pathways. Muscle contractions are fueled by adenosine triphosphate (ATP), which is produced from lipids, carbohydrates, and amino acids. When oxygen supply to the muscles is constant, during periods of steady exercise, lipids are oxidized in the Krebs cycle, and ATP is produced. When oxygen supply is limited, during periods of rapid, short-term exercise, ATP is synthesized from carbohydrates, such as glycogen, via glycolysis. This way, lipid reserves can be used for periods of normoxia, and glycogen stores can be saved for temporary periods of hypoxia. Anaerobic glycolysis requires nicotinamide adenine dinucleotide (NAD+) to produce ATP; NAD+ is available via the

following reaction:

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

Lactate dehydrogenase (LDH, E. C. 1.1.1.27) is the enzyme involved in this reversible reaction (Cahn et al., 1962; Kaplan and Cahn, 1962; Dawson et al., 1964). There are five LDH isozymes, and each isozyme is a tetramer consisting of two types of subunits, H and M. Heart muscle has mostly LDH-1, thus LDH-1 is comprised of H subunits. Skeletal muscle has mostly LDH-5, thus LDH-5 is comprised of M subunits. LDH-2, 3, and 4 consist of intermediate ratios of the subunits. Most tissues have a combination of the five isozymes (Kaplan et al, 1960; Cahn et al., 1962; Muller and Baldwin, 1978).

LDH-5 mainly converts pyruvate to lactate in the skeletal muscle, and is not inhibited by high pyruvate concentrations. Lactate is then transported by the blood to the heart muscle, where LDH-1, which is inhibited by high concentrations of pyruvate, converts lactate to pyruvate. Pyruvate is then oxidized in the Krebs cycle and ATP is produced (Dawson et al., 1964; Kaplan, 1964; Andersen, 1966; Lehninger, 1982). As a result of these differences in isozyme properties, heart muscle can maintain aerobic metabolism, while skeletal muscle can resort to anaerobic metabolism during hypoxia (Cahn et al., 1962; Dawson et al., 1964; Kaplan, 1964; Kaplan et al., 1968; Markert and Masui, 1969; Hochachka and Storey, 1975; Baldwin et al., 1978; Castellini and Somero, 1981).

Not only is the type of LDH isozyme present in the tissue indicative of hypoxia tolerance, but the total activity of LDH in the tissue can indicate its metabolic tolerances. If the total LDH activity is relatively high, due to a high activity of LDH-5, the tissue can, most likely, metabolize anaerobically (Fine et al., 1963; Pesce et al., 1964; Castellini et al., 1981).

Muscle tissues of comparative functions of diving and non-diving animals show dramatic differences in myoglobin concentrations and LDH isozyme patterns and activities. Pinnipeds (Blix et al., 1970; Storey and Hochachka, 1974; Behrisch and Elsner, 1980), cetaceans (Blessing, 1972; Castellini et al., 1981), and aquatic reptiles and amphibians (Gatten, 1985; Grigg et al., 1986) have been compared with rodents (Epstein et al., 1964; Castellini et al., 1981), primates (Vesell, 1965; Hochachka and Storey, 1975), ungulates (Pesce et al., 1964; Blix et al., 1975), and terrestrial carnivores (Vesell and Pool, 1966; Castellini et al., 1981). Within Class Aves, some skeletal muscles of diving birds--such as penguins--have higher myoglobin concentrations (Weber et al., 1974; Mill and Baldwin, 1983) and higher ratios of LDH-M to LDH-H isozymes (Wilson et al., 1963; Markert and Masui, 1969; Deshpande et al., 1984) than skeletal muscles of non-divers--such as chickens (Cahn, 1964; Pesce et al., 1964).

A comparison of the same muscle tissues of diving and non-diving individuals of the same species is also instructive (Markert and Masui, 1969; Weber et al., 1974). This comparison includes possible genetic and behavioral, as well as physiological, adaptations to hypoxia. The same tissues can be compared from the non-diving to diving stages of

maturation (Weber et al., 1974). Also, different tissues can be compared at the same stages of diving maturation (Markert and Masui, 1969; Weber et al., 1974).

Birds of the family Alcidae, which includes Murres, Murrelets, Puffins, Auklets, Razorbills, Dovekies, and Guillemots, are especially adapted to the marine environment. Alcids breed along coastlines during late spring and summer, and they winter at sea; they are primarily pelagic. They dive for their food, using their wings for propulsion, but little is known of their pelagic diving habits. Thoreson and Booth (1958), Drent (1965), Cody (1972), Sealy (1975), Asbirk (1979), Cairns (1981), and Rasmussen (1983), have studied aspects of the breeding and behavioral biology of Alcids. Follet and Ainley (1976) have described prey items of Pigeon Guillemots (Cepphus columba) during their nesting season, and Piatt and Nettleship (1985) have accumulated diving depths of several species of Alcids from gill-nets inshore and offshore. These depths range from 50 meters (m) for Black Guillemots (Cephhus grylle) to 200 m for Common Murres (Uria aalge). Piatt and Nettleship (1985) have also recorded dive durations of 112 seconds (s) for Black Guillemots diving in water 35-45 m deep, and Pigeon Guillemots have been observed to dive for 20 sec in water less than 10 m deep (personal observation). As of now, few studies have focused on the possible physiological or biochemical adaptations of Alcids to hypoxia induced by diving. (Bradley and Threlfall, 1974; Kostelecka-Myrcha, 1987).

The diving species in this study is the Pigeon Guillemot (Cepphus columba), which breeds along the west coast of North America. Pigeon Guillemots reach sexual maturity at approximately 3 years of age, and

are sexually monomorphic. They feed on benthic fish and invertebrates, and eat a wide variety of prey items (Thoreson and Booth, 1958; Drent, 1965; Follet and Ainley, 1976). Nest sites are typically located in crevasses and crannies on slopes and cliffs on the coastline (Thoreson and Booth, 1958; Drent, 1965). Man-made structures, such as three-sided cubicles formed by the supporting beams of bridges (Hodder and Graybill, unpublished observation) and abandoned piers (Hodder and Graybill, 1985), also provide nest sites. Both male and female parents feed the chicks at the nest sites. The nestling Pigeon Guillemots are active; however, they do not swim or dive. The adults abandon the nest site a few days before the young fledge, approximately thirty to forty days after hatching, and move offshore. The fledglings leave the nest site and begin diving and feeding as they also move offshore (Thoreson and Booth, 1958; Drent, 1965).

In this thesis, I have asked the following questions in regards to the muscle adaptations of the chick, fledgling, and adult Pigeon Guillemot. First, does the total concentration of myoglobin increase in the heart and pectoral muscles with maturation of the chick? Second, is there a change in the composition of myoglobin polypeptide chains expressed at the three stages of maturity? Third, does the total activity of LDH increase in the heart and pectoral muscles as the chick matures to adult? Finally, do the ratios of isozymes in these two muscles change between stages of maturation?

I also measured several blood parameters to compare Pigeon Guillemot chick, fledgling, and adult blood with that of other diving and non-diving animals.

The findings of this study shall contribute to an understanding of the total dive response of Pigeon Guillemots, as well as other diving birds.

CHAPTER II

MATERIALS AND METHODS

Animal Collection

Six Pigeon Guillemots were collected for this study: two adults and two chicks were collected from the Charleston Bridge in Charleston, Oregon, on June 1 and June 29, 1986, respectively; and two fledglings were collected from Sitka Dock in Empire, Oregon, on August 3, 1986 (Table 1, p. 9). Pigeon Guillemots use the supporting beams under these structures as nest sites, which are accessible by boat during high tide. The birds were netted, then killed by quick raps to the skull with a club within approximately 2 minutes (min) of netting. The chest cavity was opened immediately after death, and blood was collected into a heparinized syringe from the heart and aorta. The blood samples and entire bodies were then placed on ice. Upon return to the lab, the blood samples were immediately prepared; the birds were completely dissected; and the heart and pectoralis muscles were removed and stored frozen ($4^{o}C$) until used.

The ages of the chicks and fledglings were estimated from a growth curve. The curve was obtained from averaging nestling weight versus nestling age data from two years, 1982 and 1983, from nest sites at Sitka Dock (Hodder and Graybill, unpublished observations). (Appendix A.)

TABLE 1. Age, Sex, and Weight of the Six Pigeon Guillemots Collected for This Study

Collection Date	Maturation Stage	Sex	Weight (g)	Age (days)
6/01/86	adult (1)	male	439	unknown
6/01/86	adult (2)	male	497	unknown
6/29/86	chick (1)	female	105	5
6/29/86	chick (2)	male	196	9
8/03/86	fledgling (1)	unknown	481	32
8/03/86	fledgling (2)	female	506	36

Blood Parameters

Hematocrits (Hct) were determined in microcapillary tubes (Sherwood) spun in an IEC microcapillary centrifuge for 3 min. The hemoglobin (Hb) concentration of whole blood was measured spectrophotometrically after conversion to cyan-met hemoglobin (Drabkin and Austin, 1932; Kampen and Zijlstra, 1961, 1965). Ten microliters of whole blood were added to 3 milliliters (ml) of Drabkins reagent and mixed, then potassium cyanide (KCN) and potassium ferricyanide ($K_3Fe(CN)_6$) were added to convert the blood solution to the cyan-met form. The absorption (A) of cyan-met hemoglobin was read at 540 nanometers (nm) on a Zeiss PM QII spectrophotometer, and hemoglobin was determined from the relationship: Hb concentration in grams (g) per 100 ml = A_{540} x 36.4. The oxygen-carrying capacity (O_2 cap.) of whole blood was calculated from the relationship: oxygen capacity (g/100 ml) = Hb x 1.368 (Dijkhuizen et al., 1977).

Myoglobin

Preparation of Myoglobin Extract

Approximately 0.1-0.5 g of frozen muscle tissue was homogenized in 3.0 ml ice cold, 0.1 molar (M) sodium phosphate buffer, pH 7.4, for 3 min in a Waring blender. The homogenate was centrifuged at 12,000 g for 30 min in a refrigerated centrifuge (RC2-B Sorval). The supernatant was decanted and saved; the pellet was rehomogenized and centrifuged a second time as described above. The supernatants were then combined to form the crude extract.

Muscle tissue can contain hemoglobin as well as myoglobin. In order to measure only myoglobin concentration in the crude extract, the hemoglobin was precipitated out of the extract according to the procedure suggested by Weber et al. (1974). Solid ammonium sulfate was added to the crude extract, while stirring, to 65% of saturation, in order to precipitate the hemoglobin. The supernatant (containing myoglobin) was decanted, and ammonium sulfate was then removed from the myoglobin solution by dialysis against a 0.1M solution of ammonium bicarbonate, pH 8.0.

To test the effectiveness of this procedure, the precipitate (containing hemoglobin) was resuspended in the buffer described above. Then both the hemoglobin precipitate and myoglobin supernatant were assayed by column chromatography (Sephadex G-75 gel, 2x92 cm) and spectrophotometry (Zeiss PM QII spectrophotometer). The resulting absorption peaks (measured at 540 nm) from the two fractions ensured that mostly myoglobin remained in the supernatant fraction, and that

little myoglobin was precipitated out by the ammonium sulfate.

The dialyzed sample was then concentrated (Centricon-10 microconcentrator) and used for myoglobin assays and electrophoresis. (Repetitions of assays were performed on each extract preparation when possible.)

Myoglobin Assay

The concentration of myoglobin was measured spectrophotometrically as cyanmet-myoglobin. KCN and $K_3Fe(CN)_6$ were added to the sample to convert the myoglobin to cyanmet-myoglobin. The absorbance of the cyanmet-myoglobin was read at 540 nm (Reynafarje, 1963) with a Perkin-Elmer Double Beam spectrophotometer and recorder. A millimolar extinction coefficient of 11.0 and a molecular weight of 17,000 for myoglobin (Van Assendelft, 1970) was used to calculate the myoglobin concentrations, which is expressed in g/100 g wet weight of tissue.

Myoglobin Electrophoresis

Polyacrylamide gel electrophoresis (PAGE), and PAGE in the presence of sodium dodecyl sulphate (SDS-PAGE), separate myoglobin from other proteins, as well as the myoglobin polypeptide chains from each other. Two methods--7.5%-PAGE with pH 8.9 buffer (Davis, 1964) and 14% SDS-PAGE with pH 8.3 buffer (Laemmli, 1970)--were compared to determine which gave the most complete separation of the myoglobin polypeptide chains. The gels were run at 35 milliamps (mA) for approximately 2.5 hours at room temperature; stained overnight with Coomassie blue R250 in isopropanol-acetic acid, the stain described by

Fairbanks et al. (1971); then destained with 10% acetic acid (Fairbanks et al., 1971). The 7.5%-PAGE did not resolve the polypeptide chains as well as the 14% SDS-PAGE; thus, 14% SDS-PAGE was used for subsequent separations and for qualitative purposes only.

Lactate Dehydrogenase (LDH)

Preparation of LDH Solution

The procedure for preparation of the crude LDH extract was the same as the procedure for the preparation of crude myoglobin extract

Enzyme Assay

LDH activity in solution was determined spectrophotometrically from the rate of oxidation of NADH, as pyruvate was reduced to lactate (Pesce et al., 1964). The reaction medium in the cuvette (total volume of 3.0 ml) contained the following: 0.3M pyruvate; 2.13 millimolar (mM) NADH; 0.1M sodium phosphate buffer, pH 7.4; and diluted enzyme sample. The change in absorbance of the reaction medium at 340 nm was measured with a Perkin-Elmer double-beam spectrophotometer, using a mM extinction coefficient of 6.22 for NADH (Windholz, 1983). The absorption reading was valid even in the presence of other substances in the cuvette, since the other substances had an absorption of zero at 340 nm.

Enzyme activity was expressed in International enzyme units (IU/g wet weight of tissue/ml) (Dixon and Webb, 1964). Pyruvate concentrations ranging from 1.00×10^{-2}M to 1.00×10^{-5}M were used to determine the maximum velocity (Vmax) of the enzyme reaction in the

tissues (Bergmeyer, et al., 1963). The Michaelis constants (Km values) of the enzyme in the various tissues were determined by Lineweaver-Burk plots (Dixon and Webb, 1964). Km values represent the concentration of substrate (pyruvate) at which the enzyme reaches half of its maximum velocity. They provide some measurement of the rate at which pyruvate is converted to lactate.

Electrophoresis

LDH isozymes were resolved with 5.5%-PAGE (Dietz and Lubrano, 1967). The gels were run at 35 mA for approximately 2.5 hours at room temperature or 4°C, then stained from 45-60 min at room temperature, with the stain described by Markert and Masui (1969). The isozymes were separated with equal resolution at both temperatures.

The extract preparations were subjected to electrophoresis in this manner repeatedly, and the relative darkness of the bands was compared by visual inspection only.

Statistical Analyses

Where applicable, 95% confidence intervals, according to the t-test, were calculated for statistical analyses (Snedecor and Cochran, 1980).

CHAPTER III

RESULTS

Hemoglobin

The fledglings and adults had hematocrits, hemoglobin concentrations, and oxygen carrying capacities approximately 2-3x higher than those of the chicks ($p < 0.050$). There were no significant differences between the fledgling and adult blood parameters (Table 2).

TABLE 2. Hematocrits, Hemoglobin Concns, and Oxygen Capacities of Chick, Fledgling, and Adult Pigeon Guillemots (± Stand. Dev.)

Stage of Maturation	Hct (%)	n	Hb (vol %)	n	O_2 cap. (vol %)	n
Chick	15.34 ± 4.66	5	6.62 ± 2.98	8	9.06 ± 4.09	3
Fledgling	46.64 ± 6.91	8	16.00 ± 2.20	8	21.89 ± 3.01	8
Adult	50.70 ± 0.74	4	17.70 ± 3.75	4	24.21 ± 5.13	4

Myoglobin

Concentration

Table 3 shows the myoglobin concentration of the heart and pectoralis muscle tissues for the three age groups--chick, fledgling, and adult. The chick heart had more myoglobin than the chick pectoralis, and the fledgling heart had more myoglobin than the fledgling pectoralis ($p < 0.050$). There was no significant difference,

however, in the myoglobin concentration of the heart and pectoralis muscles of the adult. The concentration of myoglobin was greater in the adult heart and pectoralis than in the two younger stages ($p < 0.050$).

TABLE 3. Myoglobin Concns (g/100 g Wet Weight) in Heart and Pectoralis Muscles of Pigeon Guillemot Chick, Fledgling, and Adult (n=5)

Stage of Maturation	Heart	Pectoralis
Chick	.32 ± .04	.14 ± .01
Fledgling	.20 ± .03	.14 ± .04
Adult	.71 ± .11	.65 ± .14

The chick and fledgling pectoralis muscles appeared light red; the adult pectoralis was dark red. The heart muscles of all three stages were deep red.

Electrophoresis

No difference could be distinguished in the electrophoretic mobility of adult, fledgling, and chick heart myoglobin (Figure 1). The mobilities of the myoglobin of the adult and fledgling pectoralis were similar to one another, and to heart myoglobin, but differed from that of the chick pectoralis (Figure 2).

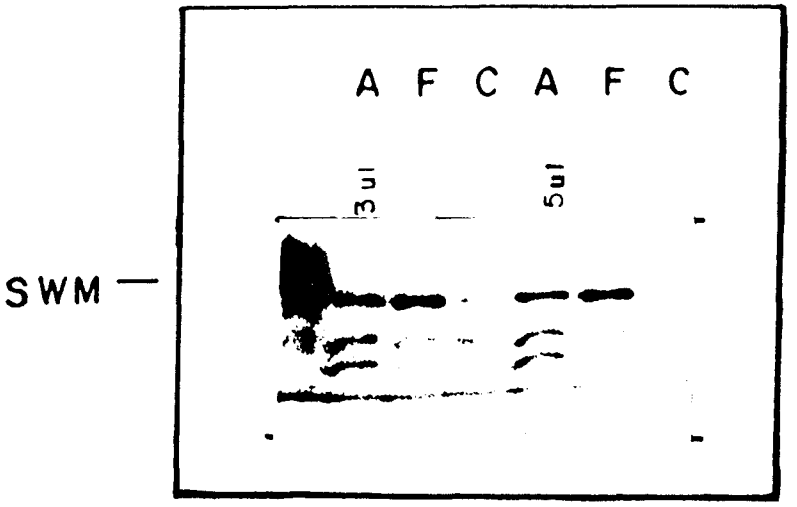


FIGURE 1. Heart Myoglobin in Pigeon Guillemot Chick (C), Fledgling (F), and Adult (A), and Sperm Whale Myoglobin (SWM). (Loading Volumes = 3, 5 microliters, (ul).)

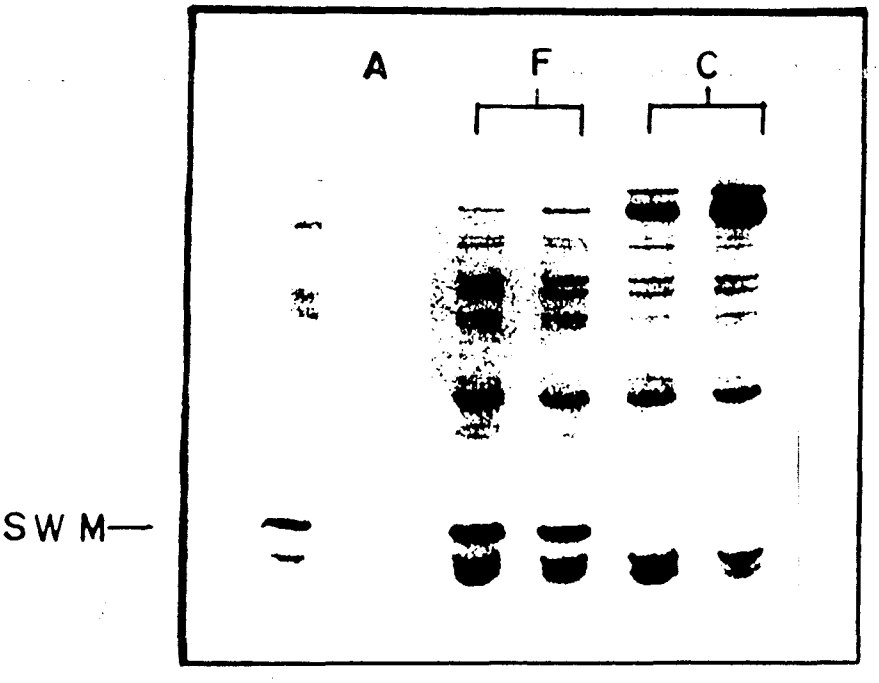


FIGURE 2. Pectoralis Myoglobin in Pigeon Guillemot Chick, Fledgling, and Adult, and Sperm Whale Myoglobin. (Loading volume = 10 ul.)

Lactate Dehydrogenase

Activity

Figure 3, p. 18, shows the LDH activities, in IU/g wet weight of tissue/ml, of the heart and pectoralis muscles for the different age groups (pH 7.4, 25°C). Both adult and fledgling had significantly higher LDH concentrations in the pectoralis muscle than the respective heart muscle ($p < 0.050$). In contrast, the LDH concentration in the chick pectoralis was not significantly higher than in the chick heart. The concentration of LDH in the heart tissue of all stages was similar. However, the LDH concentration was about twice as great in the fledgling and adult pectoralis tissue than in the chick pectoralis tissue.

Electrophoresis

Figure 4, p. 19 illustrates the electrophoretic pattern of the five isozymes of LDH. LDH-1 (H_4) is the top band seen on the gel, and LDH-5 (M_4), is the bottom band, the band which migrates farthest towards the anode. The three bands in between, from H_4 to M_4, are the following: H_4 (LDH-1); H_3M_1 (LDH-2); H_2M_2 (LDH-3); H_1M_3 (LDH-4); and M_4 (LDH-5).

All five isozymes were present in both heart and pectoralis tissues at all stages, but the heart muscle had predominantly H_4 isozymes, and the pectoralis muscle had predominantly M_4 isozymes. There was an interesting pattern in the pectoralis isozymes, however, that was not present in the heart isozymes. The heart isozymes appeared to be similar for the chick, fledgling, and adult, but the pectoralis isozymes differed amongst the three age groups (Figure 5). The chick appeared to

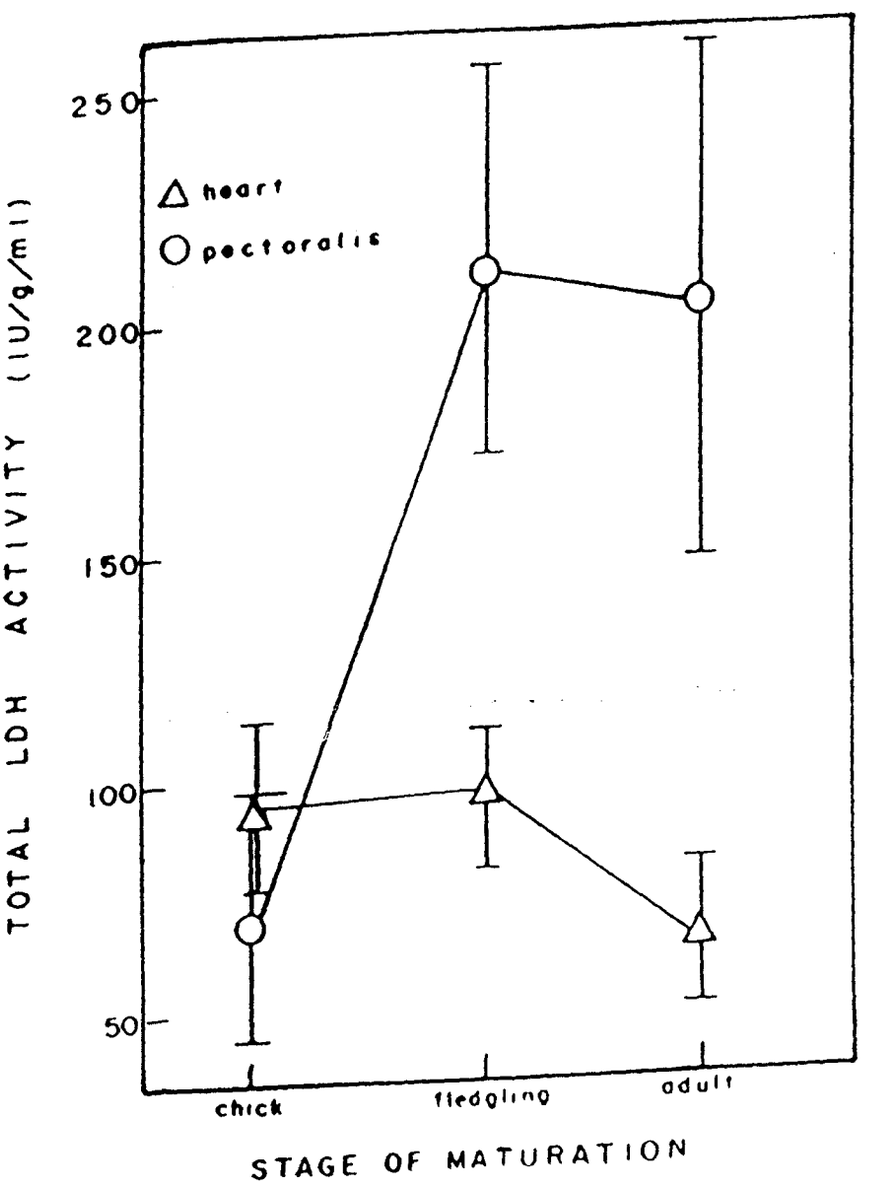


FIGURE 3. Total LDH Activity in Heart and Pectoralis Muscles of Pigeon Guillemot Chick, Fledgling, and Adult (n=5). (pH=7.4, 25°C.)

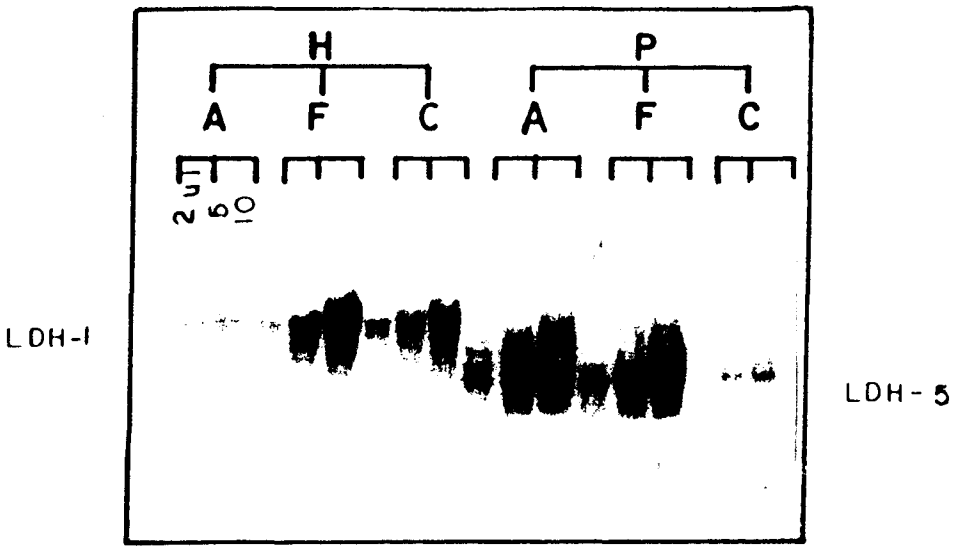


FIGURE 4. Heart (H) and Pectoralis (P) LDH Isozymes in Pigeon Guillemot Chick, Fledgling, and Adult. (Loading volumes = 2, 5, 10 ul.)

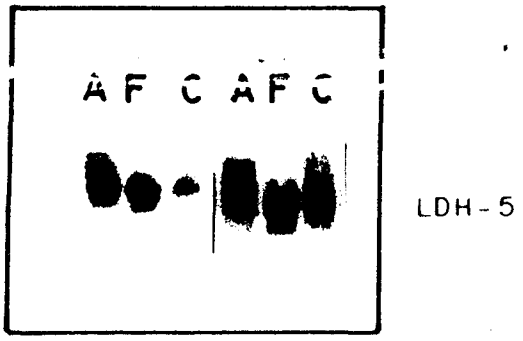


FIGURE 5. Pectoralis LDH Isozymes in Pigeon Guillemot Chick, Fledgling, and Adult. (Loading Volume = 10 ul.)

have a similar concentration of isozymes LDH-1 through LDH-4, with a higher concentration of LDH-5. The fledgling also had a strong LDH-5 band, but there appeared to be more LDH-4 than LDH-3, 2, and 1. The adult pectoralis had predominantly LDH-5, but the difference in concentration between isozyme 5 and isozymes 1-4 was not as great as for the chick and fledgling isozymes.

Enzyme Kinetics

The chick, fledgling, and adult heart enzymes all showed substrate inhibition (pH 7.4, 25°C). (Figure 6. p. 21) The pyruvate to lactate reaction rate reached a maximum velocity as pyruvate concentration increased, then the reaction rate decreased. The degree of substrate inhibition appeared to be greater in the adult heart than the fledgling and chick heart. In all stages the pectoralis LDH maintained a more constant reaction rate after maximum velocity had been obtained, and did not show the substrate inhibition that the heart muscle LDH showed.

The Km estimates for LDH, shown in Table 4, were higher for the pectoralis tissue than for the heart tissue for all stages. The Km values appeared to be larger in the fledgling and adult than in the chick for both muscle tissues.

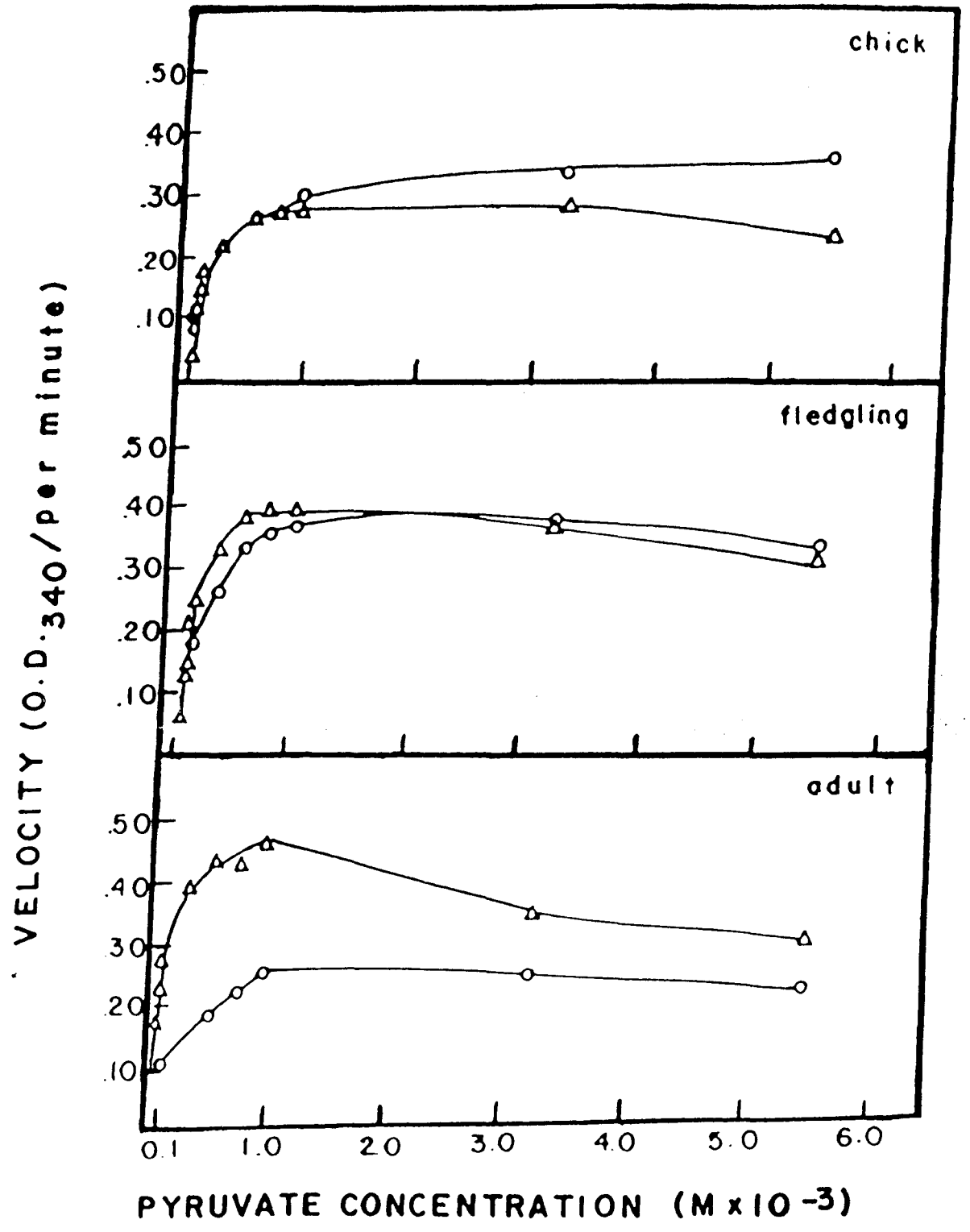


FIGURE 6. Velocity versus Substrate (Pyruvate) Concentration of LDH in Pigeon Guillemot Chick, Fledgling, and Adult (n=2). (pH=7.4, 25°C).
O=Pectoralis, Δ=Heart.

TABLE 4. Km Estimates (Pyruvate, M/L) of Heart and Pectoralis (Pect.) Muscles of Pigeon Guillemot Chick, Fledgling, and Adult.

Animal	Tissue	Range	Mean	n
Chick	heart	$5.80 \times 10^{-4} - 1.08 \times 10^{-2}$	6.51×10^{-3}	6
	pect.	$5.40 \times 10^{-3} - 3.82 \times 10^{-2}$	1.63×10^{-2}	4
Fledgling	heart	$5.75 \times 10^{-4} - 5.55 \times 10^{-2}$	2.80×10^{-2}	2
	pect.	$9.31 \times 10^{-2} - 1.38 \times 10^{-1}$	1.16×10^{-1}	2
Adult	heart	$1.07 \times 10^{-2} - 1.20 \times 10^{-1}$	6.54×10^{-2}	2
	pect.	$2.86 \times 10^{-1} - 1.05 \times 10^{0}$	6.68×10^{-1}	2

CHAPTER IV

DISCUSSION

Hemoglobin

The hematocrits and O_2 capacities of fledgling and adult Pigeon Guillemot blood are relatively high, and comparable to those of other adult Alcids (Bradley and Threlfall, 1974; Kostelecka-Myrcha, 1987). The high blood O_2 capacity of the fledglings and adults may be related to their increased activities; on the other hand, the lower hematocrit and blood O_2 capacity of Pigeon Guillemot chicks may be related to their relative inactivity at the nest site. The values for Pigeon Guillemot chicks are one half to one third those recorded for Common Murre (Uria aalge) and Dovekie (Plautus alle) chicks (Bradley and Threlfall, 1974; Kostelecka-Myrcha, 1987). Murre chicks, however, differ from Pigeon Guillemot chicks by swimming with the parent before fledging--an activity which may well be related to high blood O_2 capacity.

Blood of other diving birds, such as penguins, some ducks, and loons, has a high O_2 capacity similar to blood of fledgling and adult Pigeon Guillemots and other Alcids (Bond and Gilbert, 1958; Lenfant et al., 1969; Milsom et al., 1973; Guard and Murrish, 1975; Mill and Baldwin, 1983). Birds that maintain steady speeds during flying or fly for long periods of time, such as pigeons, swifts and hummingbirds, also have relatively high hematocrits and blood O_2 capacities (Bond and Gilbert, 1958; Palomeque et al., 1980; Johansen, 1987). Thus, diving

and sustained flight in birds appears to require similar blood carrying capacities. Shallow-diving pinnipeds and cetaceans have blood O_2 capacities similar to Pigeon Guillemot adults--values which are 2-3x those of humans (Harrison and Kooyman, 1968; Horvath et al., 1968; Lenfant et al., 1968; Clausen and Ersland, 1969; Lenfant et al., 1969, 1970; Guard and Murrish, 1975; Palomeque et al., 1980; Hedrick and Duffield, 1986). (Appendix B.)

Myoglobin

Myoglobin concentration in both heart and pectoralis muscle increases during maturation of the Pigeon Guillemot from the chick to the adult. The fledglings collected for this study were approximately 32 and 36 days of age, and the adults' age could only be approximated to at least three years old, the age of sexual maturity. The myoglobin concentration may gradually increase over maturation from fledgling to sexually mature adult, or it may sharply increase immediately after fledging, when the bird begins to dive for food.

If muscle tissue tolerance to hypoxia is correlated with muscle oxygen storage, then muscles with relatively high myoglobin concentrations can tolerate long periods of hypoxia, theroretically (Weber et al., 1974). The heart muscle in the Pigeon Guillemot adult, however, does not tolerate hypoxia, but has as much myoglobin as the adult pectoralis muscle. Perhaps Pigeon Guillemot myoglobin stores oxygen in the pectoralis muscle tissue, allowing the muscle to tolerate hypoxia, while in the heart muscle tissue myoglobin facilitates a continuous oxygen supply.

Muscle fibers can be classified into three types, according to George and Berger (1966): red, white, and intermediate fibers. Red (aerobic) fibers have large numbers of mitochondria, high oxidative enzyme activity, and high myoglobin content. White (anaerobic) fibers have fewer mitochondria, higher glycolytic enzyme activity, and low myoglobin content. Intermediate fibers have characteristics of both fibers. Although the distribution of fiber types in the muscles was not determined for the Pigeon Guillemot, the low concentration of myoglobin in the pectoralis of the chick and the fledgling suggests there are predominantly white fibers. During the progression from very light red fibers to dark red fibers in the pectoralis as the chick matures, the myoglobin polypeptides expressed first change, then increase in concentration. It is not unreasonable to suggest that this is correlated with a change from predominantly anaerobic to aerobic metabolism, and from predominantly white to red muscle fibers. The adult pectoralis muscle tissue potentially metabolizes aerobically most of the time, and relies on reserve oxygen stores only during infrequent periods of hypoxia. The heart, in all stages of maturation, however, metabolizes aerobically, based on overall muscle fiber color and unchanging myoglobin polypeptide expression through maturation.

Muscle myoglobin concentrations in both tissues of the adult Pigeon Guillemot are intermediate between those of adult pigeon and penguin skeletal muscle tissue, with adult penguin concentrations being the highest of the three (Lawrie, 1950; Weber et al., 1974; Mill and Baldwin, 1983). Pigeons may use myoglobin mainly for a continuous oxygen supply to highly aerobic tissues, but not for oxygen storage for

potentially anaerobic tissues. Conversely, penguins may use myoglobin mainly for oxygen storage for their anaerobic tissues during diving (Weber et al, 1974). Thus, the myoglobin concentration in tissues of the adult Pigeon Guillemot are higher than those of a non-diver, and lower than those of an optimal diver like the penguin. Deep-diving seals and whales and highly active rodents, such as rabbits and some bats, have the highest recorded myoglobin concentrations in skeletal muscle tissue, which is approximately 8x that of adult Pigeon Guillemots (Eichelberger, 1939; Scholander, 1940; Perkoff and Tyler, 1958; Blessing and Harschen-Niemeyer, 1969; George et al., 1971; Blessing, 1972; Ohtsu, et al., 1978; Castellini and Somero, 1981). (Appendix C.)

Lactate Dehydrogenase

The total LDH concentration (213 IU/g ± 43) in skeletal (pectoral) muscle of the adult Pigeon Guillemot is similar to that in other diving birds such as the Dabchick (Deshpande et al., 1984). It is only one tenth that reported for penguins (Castellini and Somero, 1981; Mill and Baldwin, 1983) and seals (Castellini and Somero, 1981), both of which are more accomplished divers than the Pigeon Guillemot (Appendix D). On the other hand, penguin skeletal muscle LDH is approximately 3-4x that of cardiac muscle (Castellini and Somero, 1981; Mill and Baldwin, 1983); similarly, Pigeon Guillemot skeletal muscle LDH is approximately 2-3x that of cardiac muscle. This suggests that, although the total enzyme concentrations differ, skeletal and heart muscles have similar metabolic requirements in these two birds.

During maturation, the greatest increase in total LDH activity in the pectoralis muscle occurs between the chick and fledgling stage (Figure 3), the same period of time when the myoglobin protein expression changes. This increase in LDH is the result of an enhancement mostly of LDH-5, since the fledgling pectoralis has the highest ratio of LDH-5 to other isozymes. LDH-5 is less sensitive to pyruvate inhibition than LDH-1, and therefore can reduce more pyruvate for NAD^+ regeneration during periods of anaerobiosis (Dawson et al., 1964; Kaplan, 1964; Everse and Kaplan, 1973; Hochachka, 1980; Mill and Baldwin, 1983). The fledgling pectoralis then should be better able to tolerate hypoxia than the chick pectoralis. As the fledgling matures into the adult, the ratio of LDH-5 to the other isozymes decreases in the pectoralis, but LDH-5 still remains the dominant isozyme. The adult pectoralis, therefore, is capable of both aerobic and anaerobic means of metabolism. Heart muscle from all stages of maturation has mostly LDH-1, which primarily functions to oxidize lactate and recycle pyruvate into the Kreb's cycle for ATP production. Therefore, the heart predominantly metabolizes aerobically (Dawson et al., 1964; Kaplan, 1964; Everse and Kaplan, 1973; Hochachka, 1980; Mill and Baldwin, 1983). The likely conclusion would be that the heart muscle has to function aerobically whether it is in the chick, fledgling, or adult.

During the development of Adelie penguin embryos, Markert and Masui (1969) reported a shift in isozyme expression from LDH-1 to LDH-4 and 5 in the pectoralis muscle (over a span of approximately 23 days). The heart muscle had consistent LDH-1 activity throughout development, but the other isozymes fluctuated in expression until LDH-1, 2, and 3

persisted at the end of the developmental period. They noted that although adult Adelie penguins had predominantly LDH-1 and LDH-2 in the heart, and LDH-4 and LDH-5 in the pectoralis, all isozymes were present in both muscle tissues. They speculated that:

> Tissues of diving animals might require a larger supply of the LDH isozymes at all points in the pattern, from LDH-1 to LDH-5, in order to meet physiological requirements under aerobic and also under anaerobic conditions. (p. 144)

Although they reported tissue isozyme expression only to the chick stage, their findings are comparable to the isozyme ratios found in the muscle tissues of Pigeon Guillemot young and adult birds. Yokoyama et al. (1979) reported four LDH isozymes present in the heart and pectoralis muscles of the Japanese Lesser Horseshoe bat. There was an increase in LDH-1 from embryonic through adult stages, until LDH-1 and 2 were predominant in both muscle tissues. They conjectured this increase was an adaptation to flying, an activity that places high aerobic metabolic demands on the pectoral muscle. This flying mammal, then, differs from the diving bird with regards to both LDH isozyme distribution in pectoralis muscle tissue and the metabolic demands placed on the muscle.

In the Pigeon Guillemot, the rate and timing of the shift in isozyme expression, from a high ratio of LDH-5 in the fledgling to a more even isozyme distribution in the sexually mature adult, is unknown. This shift in ratios could be gradual or sudden, similar to the increase that occurs in myoglobin concentration. The change may be correlated with fledging--perhaps the young bird can not fledge unless the total LDH activity reaches some minimum threshold of activity.

LDH in the adult Pigeon Guillemot heart muscle shows the most prominent substrate (pyruvate) inhibition (Figure 6). The enzyme reaches maximum velocity at a relatively low concentration of pyruvate, then the velocity decreases as the pyruvate concentration increases further. In comparison, the pectoralis LDH reaches Vmax at a higher pyruvate concentration than heart LDH, and the velocity does not decrease as rapidly with increasing pyruvate concentrations as the heart LDH velocity. The consequences of these differences in substrate inhibition are that pectoralis LDH can tolerate high pyruvate concentrations, enabling the tissue to resort to anaerobic metabolism during hypoxia, while heart LDH can not tolerate high pyruvate concentrations, and must rely on aerobic metabolism. This difference in substrate inhibition of the two types of LDH in tissues of Pigeon Guillemots is consistent with that reported for purified LDH isozymes from a variety of animals (Cahn et al., 1962; Wilson et al., 1963; Dawson et al., 1964; Kaplan, 1964; Pesce et al., 1964; Vesell and Pool, 1966; Kaplan et al., 1968; Storey and Hochachka, 1974; Muller and Baldwin, 1978; Suarez et al., 1986).

Michaelis-Menton constant (Km) estimates (the concentration of the substrate, pyruvate, at one-half Vmax) of LDH are typically lower for cardiac muscle than for skeletal muscle (Appendix E). Km estimates for Pigeon Guillemot LDH also follow this pattern, with estimates for heart muscle tissue approximately one-tenth those for pectoralis muscle tissue, in the fledgling and adult birds. The chick Kms, however, are lower for both tissues than the fledgling and adult, and the chick heart Km is only approximately one-half that of the chick pectoralis.

This suggests that not only do the Kms in both tissues increase as the chick matures to fledgling, but the difference in Kms between the muscles also increases during maturation. These changes in Kms correlate with the changing ratio distribution of LDH isozymes in the tissues.

Other enzyme kinetic parameters, such as turnover numbers, would be of interest as they describe the rate at which pyruvate is converted to lactate more thoroughly than the Michaelis-Menton constant (Kaplan and Goodfriend, 1964).

Summary

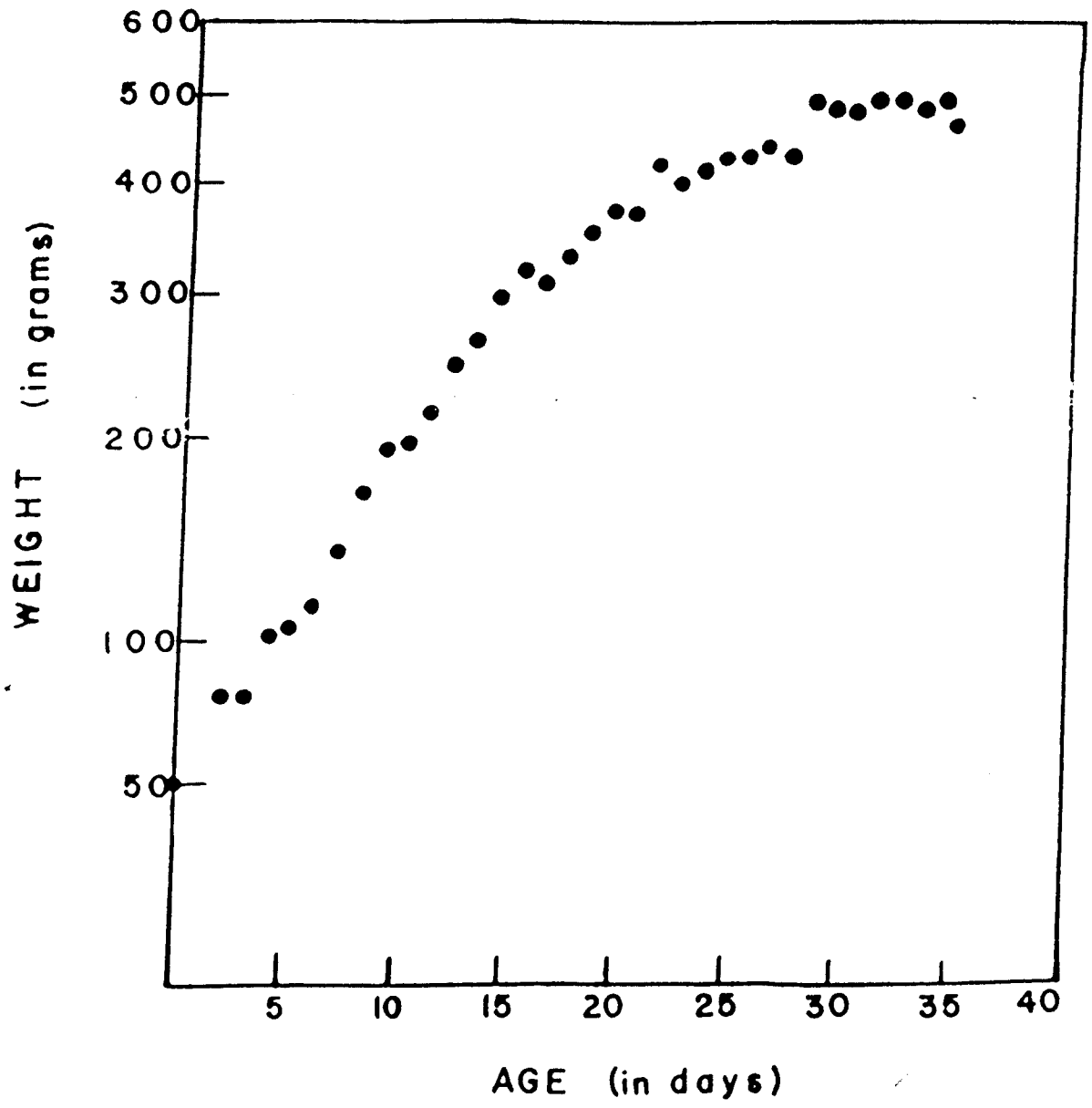
Skeletal and heart muscle myoglobin concentrations increase as Pigeon Guillemots mature from fledglings to adults. The myoglobin concentration in the adult tissues is comparable to that of other birds whose diving ability is similar to that of Pigeon Guillemots. Myoglobin polypeptide expression changes in the pectoralis muscle as the bird matures from chick to fledgling. Total LDH activity in the pectoralis muscle increases as the chick matures to the fledgling stage; then the activity remains high in the adult. The LDH activities in skeletal muscle are comparable to those found in some diving ducks, and lower than those for optimal avian divers such as penguins. The ratio of LDH-5 to other isozymes in the pectoralis muscle increases as the chick matures to the fledgling, where the highest ratio of LDH-5 to other isozymes is found. As the fledgling matures to adult, this ratio decreases, as LDH 1-4 increase in activity. The heart LDH activity and expression of LDH isozymes does not change between the three stages.

These findings suggest that the enhancement of myoglobin concentration and the glycolytic enzyme, LDH, and the increase in the ratio of LDH-5 isozyme to other isozymes, enable Pigeon Guillemots to become better metabolically adapted to exercise and diving, as they mature from chicks to adults. The heart and pectoralis muscles in the adults primarily rely on aerobic metabolism, but the pectoralis muscles can potentially resort to anaerobic metabolism during temporary periods of hypoxia.

APPENDIX A

GROWTH CURVE OF PIGEON GUILLEMOT

CHICKS (1982 AND 1983)



APPENDIX B

HEMATOCRITS, HEMOGLOBIN CONCNS, AND OXYGEN CAPACITIES OF DIVING AND NON-DIVING ANIMALS

Animal	Hct (%)	Hb (vol %)	O_2 cap. (vol %)	Source
Birds				
Alcids				
Common Murre				
chick	41			b
adult	36			b
Common Puffin	53-69			b
Dovekie				
chick	39-50	11-16	14-22	i
adult	57	17	23	i
Penguins				
chick	30	12	16	l
adult	37-53	18	25	e, j, k, l
Ducks	40	18	25	a, e
Loon	54	20	28	a
Coot	46	17	23	a
Pigeon	52	19	27	a
Pheasant and Quail	35	13	17	a
Hawk	43	13	17	a
Owl	32	8	11	a
Swift	54	18	25	m
Mammals				
Seals	31-66	13-26	12-39	c, e, f, g, j
Sea Lions	47	15	18-25	h, j
Walrus	42	16	23	j
Sea Otter	48	17	22	j
Toothed Whales	41-58	16-21	19-29	h, j, n
Rabbit	65	25	18	g
Man	44	8	11	e
Bony Fishes				
Trout	43	8	12	d

Sources: a) Bond and Gilbert (1958); b) Bradley and Threlfall (1974); c) Clausen and Ersland (1969); d) Davie et al. (1986); e) Guard and Murrish (1975); f) Harrison and Kooyman (1968); g) Hedrick and Duffield (1986); h) Horvath, et al. (1968); i) Kostelecka-Myrcha (1987); j) Lenfant et al. (1968, 1969, 1970); k) Mill and Baldwin (1983); l) Milsom et al. (1973); m) Palomeque et al. (1980); n) Ridgway et al. (1984).

APPENDIX C

MYOGLOBIN CONCNS (G/100 G WET WEIGHT TISSUE) IN MUSCLE TISSUES[a] OF DIVING AND NON-DIVING ANIMALS[b]

Animal	Myoglobin	Source
Birds		
Penguins		
adult	2.4-4.4	p, l
chick	0.1	p
Pigeon	0.3	i
Chicken	0.1	n
Mammals		
Seals	0.5-5.1	e, g, h, o
Sea lions	2.7-3.2	f, k
Walrus	3.0	k
Sea otter		
adult	3.1	f
pup	1.5	f
Sea cow (Manatee)		
pectoralis	2.5	d
heart	1.6	d
Baleen whales	0.2-6.3	f, o
Toothed whales	2.7-5.7	d, e, g
Rodents	0.1-5.6	f, j, m, n
Human	0.6-3.4	c, n

a) All muscle tissue is skeletal muscle tissue unless otherwise noted.
b) All animals are adults unless otherwise noted.
Sources: c) Andersen (1966); d) Blessing (1972); e) Blessing and Harschen-Niemeyer (1969); f) Castellini and Somero (1981); g) Eichelberger (1939); h) George et al. (1971); i) Lawrie (1950); j) Lenfant et al. (1970); k) Mill and Baldwin (1983); l) Ohtsu, et al. (1978); m) Perkoff and Tyler (1958); n) Scholander (1940); o) Weber et al. (1974).

APPENDIX D

TOTAL LDH ACTIVITIES (IU/G WET WEIGHT/ML) OF MUSCLE TISSUES[a] IN DIVING AND NON-DIVING ANIMALS[b]

Animal	LDH	Source
Birds		
Penguins		
skeletal	1,500-2,076	c, h
heart	412- 525	h
Dabchick (diver)	126- 136	e
Coot (surface feeder)	89- 98	e
Mallard	276	d
Chicken	870	d
Pheasant	542	d
Pigeon	314	d
White-crowned Sparrow	150- 400	m
Mammals		
Seals		
skeletal	1,120-1,379	c
heart	1,032	j
Sea Lions	707	c
Sea Otter	759- 801	c
Baleen Whale	257	c
Toothed Whales	1,222-1,290	c, k
Rodents	166-1,887	c, g, i
ox (beef)		
skeletal	1,016	c
heart	556	j
Bony Fishes		
red muscle fibers	617- 900	f, l
white muscle fibers	800-4,200	f, l

a) and b) Refer to Appendix C. Sources: c) Castellini and Somero (1981); d) Crabtree and Newsholme (1972); e) Deshpande et al. (1984); f) Johnston (1977); g) Messelt and Blix (1976); h) Mill and Baldwin (1983); i) Muller and Baldwin (1978); j) Murphy et al. (1980); k) Storey and Hochachka (1974); l) Suarez et al. (1986); m) Wilson et al. (1963).

APPENDIX E

Km ESTIMATES (PYRUVATE, M/L) OF CHICKEN, OX (BEEF), AND RABBIT HEART AND SKELETAL MUSCLES (ADULTS)

Animal	Muscle		Source
	heart	skeletal	
chicken	8.9×10^{-5}	3.2×10^{-3}	a
Ox (beef)	1.4×10^{-4}	1.0×10^{-4}	a
Rabbit	1.8×10^{-4}	2.8×10^{-4}	b

a) Pesce et al. (1964); b) Stambaugh and Post (1966).

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