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GENETIC VARIATION IN MITOCHONDRIAL RESPIRATORY METABOLISM IN AVIAN AND MAMMALIAN SPECIES

The University of Arizona PH.D. 1987

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GENETIC VARIATION IN MITOCHONDRIAL RESPIRATORY METABOLISM IN AVIAN AND MAMMALIAN SPECIES

by

Daniel Robert Brown

A Dissertation Submitted to the Faculty of the

GRADUATE COMMITTEE ON GENETICS

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Daniel Robert Brown

entitled "Genetic Variation in Mitochondrial Respiratory Metabolism in Avian and Mammalian Species"

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Robert G. McDaniel	4/24/87 Date
Ronald E. Allen	4-23-87 Date
Donald E. Kay	4-23-87 Date
B. L. Reid	4/23/87 Date
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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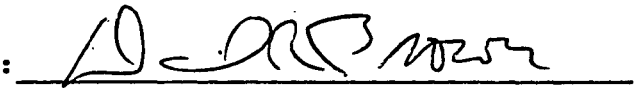
SIGNED: 

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ABSTRACT

Comparative studies documented relationships between mitochondrial respiration and phenotype among genetically divergent animals. Broiler chickens' mitochondria ranked higher than layer chickens' for state 3 respiration (16.1 $\pm$ 0.4 μM O_2/mg protein/min), respiratory control ratio (3.5 $\pm$ 0.1) and ADP:O ratio (1.05 $\pm$ 0.01 μM ADP/μA O) utilizing succinate. Broilers yielded more ($P<.05$) mitochondrial protein than did layers (7.8 vs. 6.2 $\pm$ 0.2 mg/g hepatic tissue). Within-breed mitochondrial activity had no associations with chicken weight or egg production. C57BL/6J mice mitochondria had higher ($P<.05$) respiratory control ratios (4.06 vs. 3.78 $\pm$ 0.09), and C57BL/6J females higher state 3 respiration rates utilizing succinate (strain by sex interaction $P<.01$), than A/J or BALB/cJ mice mitochondria. BALB/cJ mitochondria had higher ($P<.05$) ADP:O ratios utilizing pyruvate than C57BL/6J mitochondria (1.58 vs. 1.46 $\pm$ 0.04 μM ADP/μA O). Rapid ATP synthesis rates of C57BL/6J female mice were associated generally with reproductive superiority. Inbred mouse body weight was not associated with mitochondrial respiration. Original data confirmed $CB6F_1$, but not CAF_1, hybrid mice were heterotic for postweaning growth. Hybridization increased ($P<.05$) mitochondrial mass, but did not enhance respiration. No association existed between growth

heterosis and mitochondrial function in hybrid mice. Unexpected sexual dimorphism in mitochondrial respiration existed across mouse strains. Testosterone administration and orchiectomy confirmed female mice exhibited superior organelle activity. Mitochondrial ATP synthesis rates of Holstein cows correlated (+.30 to +.48) with milk production traits of cows and their dams suggested maternal breeding values for milk yield might be improved by considering mitochondrial effects. No breed differences in state 3 respiration (17.7 $\pm$ 0.6 μM O_2/mg protein/min) or ADP:O ratio with succinate (1.17 $\pm$ 0.01 μM ADP/μA O) existed among Angus, Brangus and Hereford cows. Regression analyses of growth traits of cows and their relatives on mitochondrial parameters revealed mainly nonsignificant mitochondrial effects. Significant regression coefficients (-0.43 to -1.05) were found for some cattle growth traits regressed on ADP:O ratios. No mitochondrial complementation occurred between neutrally-combining animal strains. Complementation occurred in mitochondrial mixtures from females of mouse strains with positive combining ability exclusively. Cow mitochondrial mixtures had lower ($P<.05$) state 3 rates than predicted.

INTRODUCTION

Traditional animal breeding has reached the status of a mature science, adequately serving the current needs of the livestock industry. Future genetic improvement of livestock and poultry will be achieved by utilizing presently untapped sources of genetic variation. These potentially include the cytoplasmic mitochondrial genome and its interactions with the nuclear genome. These potential cytoplasmic genetic effects, though long recognized, are virtually ignored in current animal breeding applications.

Distinctions between genetics and physiology are becoming blurred, with the rise of the science of molecular biology. Large gaps still exist in understanding the cascade of events leading from individual gene function to expression of quantitative traits of animals. Mitochondria, with their small genomes and intuitive potential effects on phenotype, are unique systems for study of such relationships.

The present study consisted of comparisons of respiratory capacity of intact mitochondria of genetically divergent strains within three animal species. The approach used was in vitro measurement of respiratory activity of isolated mitochondria. Associations of

mitochondrial function with phenotypic traits of economic agricultural importance were evaluated. The general rationale was that cellular energy synthesis capacity should be indicative of an animal's ability to carry out energy-dependent syntheses of products such as muscle, milk or eggs. Beef cattle, dairy cattle and chickens were economic species studied. The effects of hybridization, and potential relationships of mitochondrial function with heterosis, were assessed. Highly genetically standardized mice were studied. The value of in vitro mitochondrial complementation as a tool for predicting combining ability was investigated. These experiments were expected to help describe genetically divergent animals at a basic level of organization, and to elaborate associations of subcellular energetics with ultimate phenotypes.

LITERATURE REVIEW

The biochemistry of mitochondria is currently an extremely active field of research. However, comparative studies of genetic variation in animal mitochondrial function have not been numerous. This section describes mitochondrial biology pertinent to the present study, and previous comparative studies of plant and animal mitochondrial variation which justified this undertaking. Complementation, another potentially useful feature of genetic variation linked to mitochondrial function, is also reviewed in this section.

Mitochondrial Biology

Mitochondria carry out many metabolic functions which may ultimately affect organismal phenotype at cellular and higher levels of organization (Tzagaloff 1982). These include all of the functions associated with their own "growth" and "reproduction", such as nucleic acid, protein and membrane syntheses. Possibly more directly affecting cellular phenotype are their functions linked to provision of cellular energy supply. These include metabolism of Krebs cycle intermediates and fatty acid beta-oxidation. Yeast and plant mitochondria have large mitochondrial genomes and apparently are responsible for a wider range of metabolic activities than animal

mitochondria (Ernster and Schatz 1981). Mechanisms for nuclear control of mitochondrial metabolism are beginning to be elucidated in yeast (Woodward, Edwards and Flavell 1970; Leenders et al. 1974; Guarente 1983), and the reverse, mitochondrial influence on nuclear gene expression, has also recently been observed (Parikh et al. 1987).

Intergenomic relationships in mammalian cells remain more obscure. The mammalian mitochondrial genome (Bibb et al. 1981; Anderson et al. 1982) is highly condensed, consisting of genes for two mitochondrial ribosomal ribonucleic acids (RNAs), 22 mitochondrial transfer RNAs, a peptide subunit of adenosine triphosphatase (ATPase), several peptide subunits of the cytochrome oxidase complex, apocytochrome b, and several currently uncharacterized peptide sequences (URFs) which are thought likely to be other subunits of respiratory chain complexes. Function of these gene products seems likely to be highly constrained, though mutations in mitochondrial ribosomal RNA or ATPase may give rise to functional mitochondria varying in antibiotic resistance. The genome also includes an origin of replication, where sequence variation has been most often observed. Since a mitochondrion has been estimated to contain up to 400 structural and catalytic proteins (Tzagaloff 1982), nuclear coded genes are most likely responsible for observed

variation in animal mitochondrial morphology and metabolism (Kolata 1985).

A unique feature of mitochondrial genetics is the apparently exclusively maternal transmission of the mitochondrial genome through oocyte cytoplasm (Hutchinson et al. 1974). However, potential for paternal contribution exists via mitochondria in the midpiece of penetrating spermatocytes (Bahr and Engler 1970; Szollosi 1965). This could permit conditions necessary for mitochondrial genetic recombination, or create cytoplasmic heterogeneity on which selection pressures favoring mixed populations might act. For the majority of cells in an animal body, throughout the lifetime of the animal, mitochondria are replicated and function under influences of nuclear genes, to which the paternal parent presumably makes an equal contribution. Variation in mitochondrial function therefore need not be characterized as strictly maternal effects.

Heterogeneity for different mitochondrial characteristics has been detected within single cells (Adoutte and Beisson 1972; Coon 1978), among different tissues of a single animal (Kun and Volfin 1966; Coote, Szabados and Work 1979), and among animals of a population (Avise, Lansman and Shade 1979; Solignac et al. 1984). Differentiation during ontogeny is known to occur, but is poorly understood (Pollak and Sutton 1980; Hecht and Bradley 1981). Predictions of the fate of heterogeneous

populations of mitochondria, based on stochastic processes, have been that mixed populations will rapidly segregate to homogeneity (Michaelis 1967; Takahata and Maruyama 1981; Birky 1983; Birky, Maruyama and Fuerst 1983; Flick et al. 1985). These models have not considered that selective forces may exist favoring maintenance of heterogeneous mitochondrial populations, though there is currently only speculation that this may occur (Faust 1971; McDaniel 1984). Examples of characteristics for which mitochondrial heterogeneity has been described include deoxyribonucleic acid (DNA) sequence variation (Francisco and Simpson 1977, and many others), specific enzyme activities (Reith and Schuler 1972; Blednov, Panchenko and Shuppe 1974), antibiotic resistance (Bunn, Wallace and Eisenstadt 1974), phosphorus to nitrogen ratio (Caspari 1956), protein synthesis rates (Wagner 1972; Yatscoff et al. 1978), buoyant density (Pollak and Munn 1969) and morphology (Hackenbrock et al. 1971).

Variation in mitochondrial respiratory metabolism linked to energy transduction has also been well documented (Table 1). Parameters of respiratory metabolism assessed in the present comparative study included steady state oxygen consumption rates under different conditions, coupling of oxygen consumption to oxidative phosphorylation, and efficiency of oxygen utilization for adenosine triphosphate (ATP) synthesis. Chance and Williams (1955)

described the five steady states of mitochondrial respiration. Under in vitro conditions, state 1 is respiration (oxygen uptake) in the absence of exogenous adenosine diphosphate (ADP) and exogenous Krebs cycle substrate. State 2 is respiration with added ADP but substrate-limited. In state 3, during which net ATP synthesis occurs, both ADP and substrate are present in unlimiting amounts. State 4 is the condition when excess substrate is present but ADP is limiting. State 5 describes conditions when oxygen itself is the limit to respiration. States 3 and 4 were the steady states analyzed in the present study. The values they may assume have no theoretical limits. State 3 is easy to conceptualize; for fixed efficiencies of phosphorylation, it is conceptually equivalent to the maximum rate of ATP synthesis. State 4 has been shown not to be respiration uncoupled from oxidative phosphorylation, but instead oxidative phosphorylation utilizing endogenous ADP generated from ATP by ATPase (Bishop and Atkinson 1984), sort of an "idling" rate. Integral factors affecting steady states include net capacities of beta-oxidation, Krebs cycle and the electron transport chain, and relative pools of ADP, oxygen, and reduced nicotinamide adenine dinucleotide (NAD) or flavin adenine dinucleotide (FAD). The parameter of respiratory control ratio is estimated as the ratio of state 3 to state 4 rates (Estabrook 1967), and

so indicates the degree of coupling of respiration to oxidative phosphorylation. Disproportionate variation in either state 3 or state 4 rates will affect respiratory control ratios. Respiratory control ratio has a theoretical low value of 1, i.e. respiration rate totally insensitive to ADP concentration, and no upper bound. In vitro respiratory control ratios which are "low" may be indicative of mitochondria damaged during isolation, but if the concept of variability for this parameter is accepted at all, above some subjective level of acceptability respiratory control ratio is not a reflection of damage. Integral factors affecting respiratory control, for uniform state 3 capacity, include passive permeability of substrates and adenine nucleotide transport (ADP import) capacity (Tarjan and Von Korff 1967; Groen et al. 1982; Jacobus, Moreadith and Vandegaer 1982). The efficiency of oxygen utilization during ATP synthesis (ADP:O ratio) has been considered by basic researchers to have fixed theoretical values, differing only between NAD-linked and FAD-linked substrates (Kadenbach 1986). If oxidative phosphorylation were perfectly coupled to electron flow, classical theoretical ADP:O values were assumed to be 2.0 (FAD-linked) and 3.0 (NAD-linked) molecules of ATP formed per atom of oxygen reduced (i.e. per pair of electrons passing down the chain) (Estabrook 1967). Recent experimental studies have demonstrated the number of ATP molecules formed per pair of

electrons passing a coupling site is not necessarily equal for each of the three coupling sites in the chain, and that a fixed value of 2.67 for NAD-linked substrate may be an upper bound (Brand et al. 1978). As for respiratory control, in vitro ADP:O ratios of damaged mitochondria will be low, but above some subjective level, observed values other than the fixed theoretical value must be assumed to reflect true differences in efficiency of oxygen utilization. In fact, ADP:O ratios much larger than the theoretical limits have been experimentally observed, suggesting passage of fewer than two electrons past a coupling site could be sufficient to form an ATP molecule in those mitochondria. Sarkissian and Srivastava (1969) reported observing ADP:O ratios as high as 5.8 with alpha-ketoglutarate and 3.4 with succinate with mitochondria of wheat hybrids. The unusually high ADP:O ratios might be explained by invalid assumptions about oxygen concentration of the reaction medium. For example, if oxygen concentrations were higher than assumed, ADP:O ratios could be misconstrued to exceed theoretical limits. However under usual conditions, comparing large numbers of samples, it is impractical to determine precise oxygen concentrations.

Mitochondrial Respiratory Metabolism and Economic Traits

Plants

The possibility that variation in mitochondrial respiratory capacity might underly or be associated with phenotypic traits of economic importance has been most extensively studied in agronomic plant species. One approach has been to screen plant populations for assessment of variability of mitochondrial respiratory capacity among plants, and relationships with yield characteristics. Significant variation among individual plants, and associations of mitochondrial respiratory parameters with seedling growth (McDaniel 1969b) and forage yield (Schneiter et al. 1974, 1976; Dobrenz et al. 1975; McDaniel et al. 1977) were found. Mitochondrial activity was thus demonstrated to be a potential tool for rapid biochemical screening for the most desirable plants. The mitochondrial screening technique would be valid whether there was a direct causal relationship between mitochondrial metabolism and phenotypic variation, or if superior mitochondrial function simply reflected overall metabolic superiority of a genotype.

A much more extensive body of work has been conducted particularly to evaluate potential relationships between enhanced mitochondrial respiratory capacity and the

phenomenon of heterosis (Sarkissian 1972; McDaniel 1973a, 1986). Although no test had been conducted which could distinguish between a causal effect of mitochondrial respiratory capacity on heterotic phenotype, and a general expression of heterosis at subcellular as well as higher phenotype, early studies suggested the two might be closely linked (Sarkissian and McDaniel 1967). As described above, mitochondrial screening might serve as a simple biochemical predictor of heterosis. Such a test might be especially useful for initial screening of hybrid genotypes before committing to extensive field tests of productivity. Significant relationships were found between mitochondrial respiratory capacity of hybrids which tended to or did exceed the theoretical midparental mitochondrial respiratory capacity (or that of the superior parent), i.e. "mitochondrial heterosis", and whole-plant heterosis for economic characteristics in several plant genera (McDaniel and Sarkissian 1968; McDaniel 1969a, 1972; Sarkissian and Srivastava 1969; Doney, Theurer and Wyse 1985). Many other studies on relationships of mitochondrial heterosis with plant growth and yield heterosis were conducted in conjunction with tests of in vitro mitochondrial interactions, as discussed below.

Animals

Discoveries of variability of mitochondrial respiratory capacity and mitochondrial heterosis associated with economic traits of plants were followed by studies on similar intuitively plausible relationships in animal species (Wagner 1972; Grunder 1974). Differences in mitochondrial activity among Drosophila strains varying in growth rate, body weight (McDaniel and Grimwood 1971) and life span (Martinez and McDaniel 1979) were observed. Raicu et al. (1970) presented comparative data indicating variation existed for qualitative mitochondrial characteristics in hamsters, but with no discussion of relationships to phenotype. Sarkissian, Nicholas and McDaniel (1968) reported mitochondrial heterosis in mouse hybrids, though it was not clear if the hybrid mice were heterotic for any other trait. Chai and Mukherjee (1974) provided essentially control data on mice of uniform phenotypes, for the traits reported, with uniform mitochondrial respiratory capacity, although the mitochondrial data were incomplete. In the first definitive study relating mitochondrial activity to phenotype of vertebrate animals, Dzapo, Reuter and Wassmuth (1973) demonstrated relationships between mitochondrial respiration rate, ADP:O ratio, and cytochrome c oxidase and succinate dehydrogenase activities, and birth weight, growth rate and kidney fat of inbred and hybrid rabbits.

Very few comparative studies on mitochondrial activity variation in livestock species have been conducted. Wolanis, Dzapo and Wassmuth (1980a,b) found significant variation in state 3 respiration rate, respiratory control ratio and ADP:O ratio among inbred and crossbred sheep. This variation appeared not to be associated with slaughter weight of the different genotypes, the only phenotypic trait discussed in the report. Significant differences among sheep genotypes were also found for specific enzyme activities representing mitochondrial fatty acid oxidation, the Krebs cycle and the electron transport chain (Wolanis, Dzapo and Wassmuth 1979). Some of these were highly positively correlated (r=.61 to .88) with the trait of weaning percentage (Wolanis, Dzapo and Wassmuth 1980b), which formally constituted documentation of correlated or causal relationships between mitochondrial function and an economic trait.

Significantly enhanced activities of enzymes of mitochondrial respiration were found in crossbred swine (Dzapo and Wassmuth 1978, 1979b, 1984; Schwalenstoecker, Dzapo and Wassmuth 1978a),: "evidence that mitochondrial oxidative energy metabolism is one of the most important manifestations of heterosis in cell physiology" (Dzapo and Wassmuth 1984). It was noted that activity of cytochrome oxidase, a product of nuclear and mitochondrial intergenomic interaction, was much more enhanced than activity

of nuclear-coded enzymes. "Highly significant heterotic manifestations" were found for respiration and oxidative phosphorylation of intact mitochondria of the crossbred swine (Dzapo and Wassmuth 1983). Enhanced mitochondrial activity of crossbred swine was putatively associated with positive heterosis for piglet survival (Dzapo and Wassmuth 1979b,c; Dzapo, Wessling and Wassmuth 1979), reproduction rate and carcass leanness (Dzapo, Schnarr and Wassmuth 1983; Schwalenstoecker, Dzapo and Wassmuth 1978b). These studies of crossbred swine constitute the most extensive exploration of mitochondrial function in a livestock species to date.

Little informative data previously existed on mitochondrial respiratory metabolism of chicken cells. Mukherjee et al. (1970) compared oxygen uptake of hepatic mitochondrial suspensions from unsexed day-old White Leghorn, White Rock, and Leghorn X White Rock hybrid chicks. Integrity of the mitochondria (respiratory control) was not assessed. Only state 1 (reducing substrate- and ADP-limited) respiration rate values were reported, probably the least informative respiratory measurement with regard to normal metabolism. No measure of variability or description of statistical analysis was reported, though uptake was indicated to "differ" between inbreds. Dziewiecki and Kolotaj (1976) compared state 4 hepatic mitochondrial respiration rate values only for 12-

15 wk old mixed-sex Leghorns, "Greenlegs", and reciprocal hybrids, utilizing different Krebs-cycle substrates. No measure of variability or description of statistical analysis was reported, though various highly statistically significant differences were claimed. A second study (Dziewiecki and Kolotaj 1980) of 2-10 mo old hens further included White Rocks and their hybrids. State 1 respiration was also reported. This study included the only reported measures of variability of mitochondrial respiratory rates of chicken cells. No relationships with other phenotypic traits were explored in these three studies. Dziewiecki and Kolotaj noted their Greenleg "hybrids manifest[ed] successful heterosis in respect of many utility traits", and that in general hybrids exhibited mitochondrial respiration rates intermediate to those of parental strains (1976, 1980). Though not discussed by the authors, there can be seen a positive relationship in the rank of mitochondrial respiration rate with body weight, with the exception of Greenleg hybrids, across these three reports.

Oxygen consumption of chickens has been studied more extensively at the individual-animal level. Though the fraction of total oxygen consumption due to mitochondrial respiration is unknown and not necessarily constant, and since previous studies of avian mitochondrial respiratory metabolism are extremely scarce, results of

mitochondrial respiration studies may at present be best interpreted in view of what is known about the whole animal. In a selection experiment, MacLaury and Johnson (1972) developed high and low oxygen consumption lines of chickens. The realized heritability of oxygen consumption was estimated to be 0.08 $\pm$ 0.01 ($P<.001$). The response to selection was asymmetric; greater in the high oxygen consumption line, with the low oxygen consumption line apparently reaching a minimum metabolic threshold. The low oxygen consumption line decreased respiration frequency six-fold in comparison to the respiration frequency increase of the high oxygen consumption line (Johnson and MacLaury 1974). Females had higher respiratory frequencies than males. Body weight of the low oxygen consumption line was significantly heavier, though no body composition was reported, while the high oxygen consumption line had higher egg production and hatching rates (Johnson and MacLaury 1973). The authors attributed these relationships to pleiotropic genetic effects. Using a different approach, Kuenzel and Kuenzel (1977) and Denbow and Kuenzel (1978) compared basal and resting metabolic rates calculated from oxygen consumption of 0-8 wk old Leghorn and broiler chicks. The basal metabolic rate of broilers (kcal/bird) was significantly higher than that of Leghorns, though after adjustment for the difference in body weights by

covariance analysis the ranking reversed. No difference in resting metabolic rate was detected.

Virtually no studies have been reported on mitochondrial respiratory variation in cattle. However, recent findings from opposite ends of the spectrum of genetics have renewed attention to mitochondria as potential determinants of variation in economic traits of cattle. A number of nucleic acid restriction endonuclease fragment length polymorphisms, apparent point mutations distributed over the mitochondrial genome, were found to exist among three breeds of dairy cattle, and also among maternal lineages of Holstein cattle (Laipis, Wilcox and Hauswirth 1982). Heterogeneity for larger insertions in the origin of replication of the mitochondrial genome within a single cow was also reported (Hauswirth et al. 1984). Interpretation of these findings by the authors concentrated on implications for differential amplification and rapid evolution of mitochondrial DNA (Hauswirth and Laipis 1982; Michaels, Hauswirth and Laipis 1982), without consideration of effects of such variation on gene product function or mitochondrial activity. The possibility that variation in mitochondrial DNA sequences might be extensive among maternal lineages suggested it might constitute a source of measurable differences among lines in phenotypic traits of economic importance. Bell, McDaniel and Robison (1985) cited differences in reciprocal crosses, higher

heritability estimates from daughter-dam regression than from paternal half-sister correlations, and better predictions of daughters' performance than of sons' progenys' performance from dams' performance, as effects that might be due to cytoplasmic genetic factors. These authors grouped performance records of pedigreed cows by cytoplasmic line, and found in statistical models that cytoplasmic origin was a significant component of variation in milk and milk fat yield. Using the same approach, Huizinga et al. (1986) found cytoplasmic line to account for up to 10% of the variation in milk fat plus protein, and up to 13% of the variation in milk returns (an economic index). The authors stated, "Some of these cytoplasmic components accounted for more phenotypic variation in reproductive traits of nulliparous heifers than most additive genetic components found in the literature", though cytoplasmic origin was not statistically significant for reproduction traits in that study. However, Kennedy (1986) has subsequently presented persuasive evidence from modeling studies, that residual additive genetic effects, unaccounted for in the methods of Bell, McDaniel and Robison (1985) and Huizinga et al. (1986), might erroneously be interpreted as cytoplasmic effects.

Mitochondrial Complementation

A phenomenon characterized by measurable in vitro interactions of artificially mixed mitochondria derived from genetically divergent sources has been termed "mitochondrial complementation" (McDaniel and Sarkissian 1966). Using different plant and animal sources, complementation, usually though not exclusively an enhancement of mitochondrial activity, has been reported for mitochondrial traits as selectively listed in Table 1. The significance of this effect was that it was claimed to parallel combining ability of source strains, and might so be useful as a predictor of combining ability. Parental strains whose hybrid progeny were heterotic for one or more traits, including mitochondrial traits, exhibited mitochondrial complementation. Strains whose progeny were nonheterotic did not exhibit mitochondrial complementation. Most studies reported results of single or few hybrids. Relationships of complementation with traits of hybrids across studies thus tended to be "rank" in nature. With larger groups of hybrids, linear correlations of complementation with grain yield heterosis as high as + 0.69 for barley (McDaniel 1972) and + 0.85 for wheat (Barratt and Flavell 1977) have been reported. However, some researchers were unable to detect or replicate complementation (Table 1). Several reviews on the topic have been

Table 1. Survey of mitochondrial complementation experiments[a].

Genus	Completion Observed: Mitochondrial Characteristics	Completion Observed: Phenotypic Characteristics	References
Zea	State 3, ADP:O	Germination, growth, grain yield	McDaniel & Sarkissian 1966, 1967, 1970
	State 3, ADP:O		Sarkissian & Srivastava 1967
Triticum	RC, ADP:O, ATPase	Sterility, growth, grain yield	Sarkissian & Srivastava 1969; Srivastava, Sarkissian & Shands 1969
	Cytochrome oxidase, NADH:cytochrome c reductase		Sarkissian & Srivastava 1971
	ADP:O		Barratt & Flavell 1977
	Unstated		Hobson 1971
Triticale	ADP:O	Unstated	McDaniel 1973b
Hordeum	ADP:O	Growth, yield	McDaniel 1972
Beta	RC, ADP:O	Root yield	Doney, Theurer & Wyse 1972
Culex	"6 mitochondrial enzyme systems"	"Teratologic phenotype"	French 1971
Drosophila	ADP:O	Growth, weight, life span	Martinez & McDaniel
	State 3, RC, ADP:O		McDaniel & Grimwood 1971
Oryctolaqus	State 3, cytochrome c oxidase	Growth rate	Dzapo, Reuter & Wassmuth 1973
Mus	"Oxidative and phosphorylative activity"	Unstated	Sarkissian, Nicholas & McDaniel 1968

Table 1 continued

Sus	State 3, ADP:0, cytochrome c oxidase, NADH: cytochrome c reductase	Birth rate, weaning rate	Dzapo & Wassmuth 1982
----------Complementation Not Observed----------			
Zea	State 3, RC, ADP:0	Growth, grain	VanGelder & Miedema 1975
	State 3, RC, ADP:0	Dry matter yield	Hanson, Moreland & Shriner 1973
Zea	State 3, RC, ADP:0, cytochrome c oxidase		Sen 1981
	RC, ADP:0		Rodgers 1976
Triticum	ADP:0	Grain yield	Sage & Hobson 1973
	RC, ADP:0, cytochrome c oxidase		Sen 1981
Hordeum	RC, ADP:0, cytochrome c oxidase	Growth	Sen 1981
	ADP:0		Ellis, Brunton & Palmer 1972
	State 3, RC, ADP:0		Zobl et al. 1972
Beta	ADP:0	Root weight	Doney, Theurer & Wyse 1975
Glycine	State 3, RC, ADP:0	Growth	Hanson, Moreland & Shriner 1973
Mus	State 1	Growth	Chai & Mukherjee 1974
Gallus	State 1	Growth	Mukherjee, Stevens & Hoogendoorn 1970

[a]RC = respiratory control ratio, ADP:0 = ADP:0 ratio.

published (Sarkissian 1972; Srivastava 1972, 1981; McDaniel 1986).

The mechanisms by which complementation might occur were unknown, except for some evidence that direct particle-to-particle contact was necessary (McDaniel 1967; McDaniel and Sarkissian 1970; Sarkissian and Srivastava 1973). Past speculations on mechanisms included donation of inhibitors/enhancers, phospholipases, or proton gradients (McDaniel 1986), or membrane conformational changes due to intermitochondrial contact (McDaniel and Sarkissian 1970). One instance of mitochondrial exchange of soluble factors (amino acid precursors) was known in yeast (Leiter et al. 1971), but this affected a trait (amino acid synthesis) not reported in complementation studies on plants or animals. Few attempts to elucidate possible mechanisms have been reported.

Regardless of mechanisms, a distinction must be made between predictions based on in vitro complementation tests and models for in vivo interactions among heterogeneous mitochondria. Intracellular heterogeneous mitochondrial populations are known to exist, whether arising by mutation or "paternal leakage" (Birky 1983). Their distributions and segregation have been modelled statistically (Takahata and Maruyama 1981). Heterogeneous mitochondria may interact in a variety of ways, including genetic recombination (Horak, Coon and Dawid 1974; Fonty et

al. 1978), which may ultimately affect cellular phenotype. However, in vitro mitochondrial complementation is not predicated on the existence of in vivo heterogeneous mitochondrial populations. Neither is a relationship with combining ability supposed to depend upon in vivo recreation of conditions occurring when mitochondria are artificially mixed (although interesting implications for in vivo microinjection experiments arise [McDaniel 1984]). There simply exists evidence for in vitro mitochondrial interactions under defined conditions, and statistically described relationships of complementation with combining ability.

Predictions of the mitochondrial complementation theory stated simply are that divergent inbred strains with positive combining ability for one or more traits are likely to show complementation, and that strains with neutral or negative combining ability are likely to show no or negative complementation. These predictions should affect choice of test systems for studies of the complementation effect. Some past animal experiments on complementation may be criticized for their choices of test systems. Chai and Mukherjee (1974), using mice, and Mukherjee et al. (1970), using chicks, failed to choose hybrids heterotic for any reported trait. Lack of observed complementation in those studies thus is in fact in agreement with complementation theory ("consistent negative").

Dzapo, Reuter and Wassmuth (1973), using rabbits, had a heterotic hybrid but no control non-heterotic hybrid, making it hard to exclude rigorously artifactual causes for observed "consistent positive" complementation. Dzapo and Wassmuth (1982), using swine, reversed the usual experimental process by making phenotypic comparisons only after grouping based on complementation results, though this would be the ultimate use of complementation testing in animals should the theory be widely substantiated.

The majority of reports supporting the existence of the complementation phenomenon come from studies on plants (Table 1). These are also subject to some criticisms. In many experiments mixtures were made on the basis of fresh tissue weight without regard to loss during homogenization or possible differences in mitochondrial yield between strains (see Results). The true contribution of each strain to mixtures was therefore not accurately assessed. Some abstracts presented no data or experimental details. Especially in some early reports, measures of variability or any statistical analyses were lacking, making objective evaluation of results impossible. In others, statistical analyses were vaguely described, or data were apparently uncritically pooled before analyses, inappropriate technique for repeated measures data obtained from respiration traces.

MATERIALS AND METHODS

In this section, basic techniques used throughout the study are described. Control experiments to document the validity of certain techniques are also described in this section. Experimental conditions for the series of trials conducted are described for each species. Techniques which were explored but not extensively applied are also discussed.

Measurement of Mitochondrial Respiratory Metabolism

The established technique of differential centrifugation (Johnson and Lardy 1967) was adapted for preparation of mitochondrial fractions. Preliminary practices were conducted using mouse hepatic tissue. Mice were euthanized by cervical dislocation[1] and the entire gall bladder-free liver excised. Hepatic homogenate was prepared with three gentle passes of a serrated-tip teflon pestle in a 30 ml hand tissue grinder. A step by step description of the procedure finally adopted for separation of mitochondrial fractions from cell lysates is given in Appendix 1. Procedures for isolation of plant mitochondria (McDaniel 1967) were found to be unsatisfactory for preparation of

1 Euthanasia of all animals sacrificed in this study was conducted according to current recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA Panel on Euthanasia 1986).

coupled animal mitochondria, particularly with regard to osmolarity of buffer and sensitivity to fatty acid contaminants of included albumin.

Isolation and reaction buffers finally adopted (Table 2) were modified from the procedure of Vercesi, Reynafarje and Lehninger (1978). Cocarboxylase, a soluble cofactor in pyruvic- and alpha-keto-acid dehydrogenation, was routinely included in the reaction buffer. The cofactor magnesium, frequently included in mitochondrial reaction buffers, was tested and found not to enhance maximally-stimulated respiration rate, respiratory control or phosphorylation efficiency. This was probably due to inclusion of ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA), which exhibits a high specificity for calcium binding preferentially to other divalent cations, rather than the commonly used (ethylenedinitrilo)-tetraacetic acid (EDTA) which may deplete endogenous mitochondrial magnesium. Exogenous cytochrome c, which has been experimentally included in reaction buffers for plant mitochondria (McDaniel 1967), was also tested, with no effect. Use of a separate solution (0.25 M sucrose, pH 7.2) for intermediate washing steps was found to be unnecessary, with grinding buffer suitable for all preparation steps and for final pellet suspension.

The Krebs' cycle substrates alpha-ketoglutarate, succinate, and a mixture of pyruvate plus malate, were

Table 2. Composition of mitochondrial buffers.

		mM	g/l
Grinding buffer	sucrose	250	85.575
	EGTA[a]	5	0.190
	HEPES[b]	3	0.715
	adjusted to pH 7.2 with KOH		
Reaction buffer	mannitol	250	45.540
	tris[c] base	10	1.211
	KH_2PO_4	10	1.361
	cocarboxylase	0.5	0.250
	adjusted to pH 7.2 if necessary		

[a] ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid

[b] N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid

[c] tris(hydroxymethyl)aminomethane

prepared at the concentration indicated for each trial by suspension in a minimum volume of distilled, deionized water and adjusted to neutral pH by addition of flakes of freshly ground crystalline potassium hydroxide. For evaluation of fatty acid oxidation, beta-hydroxybutyrate was similarly prepared. Preparation of ADP solutions was also similar. Contamination of crystalline ADP with its decomposition product AMP was tested for by high pressure liquid chromatographic separation, and stock solutions were corrected to their true ADP content. The AMP content of fresh crystalline ADP was found to be 3 - 5%.

In control experiments, effects of the succinic dehydrogenase competitive inhibitor malonate, electron transport inhibitors rotenone and antimycin A (alone and in combination with the cyanide-insensitive respiration inhibitor propyl gallate), and their solvent ethanol, on mitochondrial fraction respiratory metabolism were evaluated. Effects of ADP concentration, cumulative amount of ADP added, and mitochondrial protein concentration were also tested.

Mitochondrial respiration (oxygen consumption) and oxidative phosphorylation were monitored using a Clark-type oxygen electrode (Lessler, Molloy and Schwab 1966; Estabrook 1967). Briefly, the polarographic oxygen monitor supplys a polarizing voltage between the silver anode and platinum cathode of the electrode, via an electrolyte

solution separated from the reaction mixture by a gas-permeable teflon membrane. Catalytic reduction of dissolved oxygen at the cathode varies the polarizing voltage in proportion to the rate of diffusion of oxygen from the reaction mixture across the membrane to the cathode. The diffusion rate is proportional to the oxygen concentration in the reaction medium, and is highly sensitive to temperature. Therefore temperature of the reaction mixture was precisely maintained using a water jacket and recirculating controller accurate to $\pm$ 0.1 C. Routine reaction temperature was 25 C, since respiration at higher temperatures was extremely rapid, limiting the number of respiratory cycles measurable on a preparation before oxygen became completely depleted. Diffusion of atmospheric oxygen into the reaction mixture was prevented by a plunger (which also served to hold the electrode) precision fit into the reaction vessel. The amount of atmospheric oxygen which might have diffused into the reaction mixture via an access slot through which substrate, ADP and inhibitors were injected was negligible. The oxygen concentration of air-saturated reaction buffer, after exclusion of all air from the reaction vessel but before mitochondrial oxygen uptake, was assumed to be 240 μM (Estabrook 1967). Constant stirring with an immersed magnetic stirrer prevented a localized oxygen gradient in

the reaction medium at the surface of the electrode membrane.

For a typical respiration measurement, an aliquot of mitochondrial fraction suspension plus reaction buffer were temperature-equilibrated with stirring for air saturation for exactly 3 min. Then the plunger was inserted into the reaction vessel, all air excluded, and recording of oxygen consumption begun. Change in oxygen concentration of the reaction medium was recorded on a single-pen linear strip chart recorder. Steady state 4 respiration was initiated by introduction of an excess amount of substrate, injected with a Hamilton microliter syringe (typical volume 5 µl). Maximally stimulated respiration (steady state 3) was subsequently initiated with a pulse of ADP, injected in a 0.5 µl volume precisely delivered using a Hamilton microliter syringe fitted with a Hamilton PB600 dispenser. State 3 respiration was initiated repeatedly in cycles of oxidative phosphorylation of added ADP, and subsequent return to state 4 respiration once ADP again became limiting (Figure 1).

Each linear steady state slope (change in oxygen concentration per unit time) was calculated by manually counting 1 mm chart units over a fixed 2.5 cm X (time)-axis distance, to minimize variation due to chart measuring. Respiratory control ratio was calculated as the ratio of state 3 to immediately subsequent state 4 slopes. The

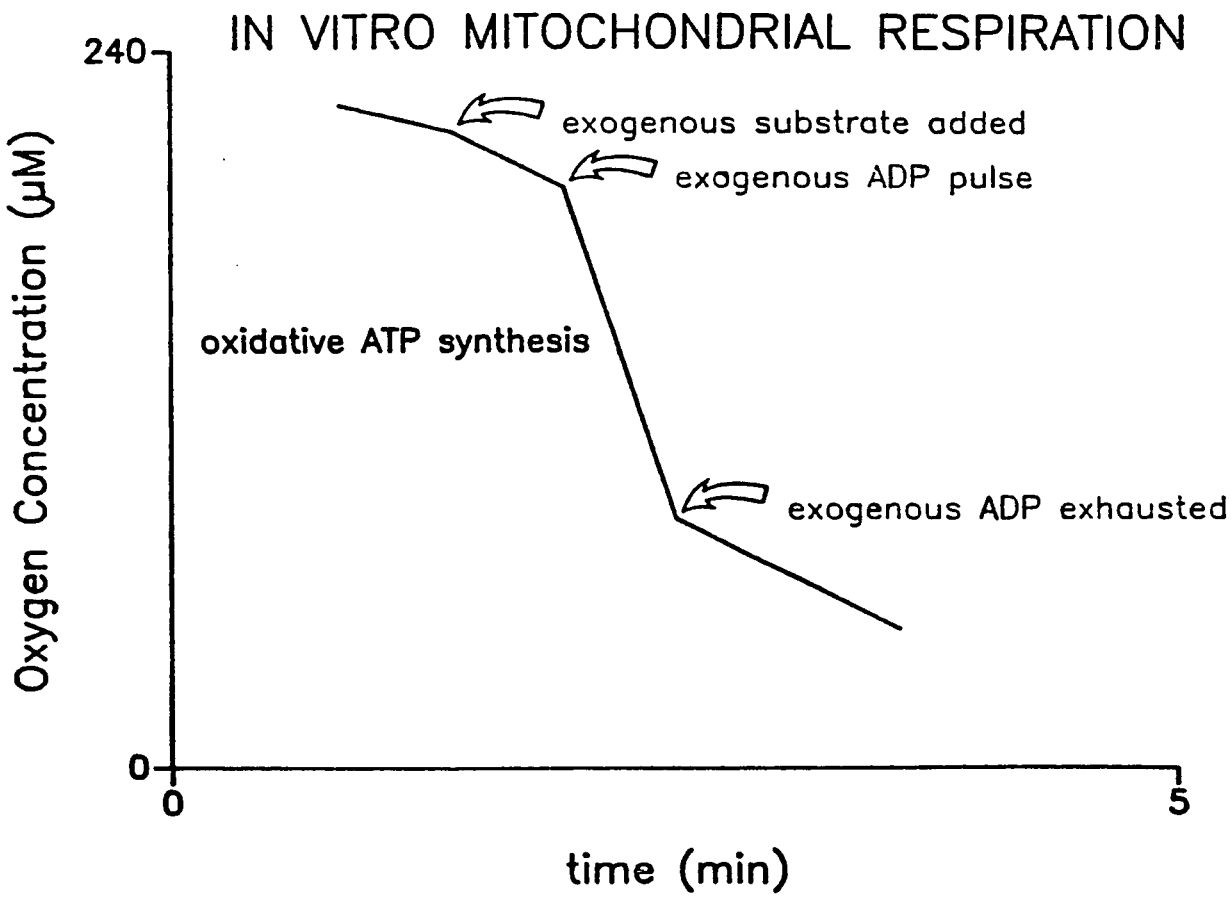

Figure 1. Polargraphic measurement of mitochondrial respiration and oxidative phosphorylation.

ADP:O ratio was calculated from the amount of oxygen consumed between the initiation of state 3 respiration by the addition of a known amount of ADP, and the return to state 4 respiration following ADP exhaustion.

Steady state respiration rates were converted to specific activities based on the amount of mitochondrial fraction protein in the reaction. Mitochondrial fraction protein was quantitated in one of three different ways. In the simplest case, as for mouse and chicken liver mitochondria, an aliquot of mitochondrial preparation was reserved for total protein quantitation immediately after the final resuspension. There was found to be an absolute requirement for inclusion of fatty acid-free bovine serum albumin (BSA) in all reaction mixtures (Weinbach and Garbus 1966), and when mitochondria were isolated immediately after tissue excision the BSA could be witheld until just before respiration measurements were made. If tissue samples were to be held for some time before mitochondrial isolation, as for transportation to the laboratory, mitochondrial integrity was best protected by immediate tissue homogenization in BSA-containing (0.5% w/v) buffer. Subsequent isolation steps were also conducted using BSA-containing buffer. For samples of this type, mitochondrial fraction protein was estimated by subtraction of the amount of BSA supplied by the final resuspension buffer volume from the total protein in the preparation. Total protein

for all samples was quantitated by the colorimetric method of Bradford (1976).

The question arose as to whether BSA was carried over from step to step in mitochondrial pellets during isolation, i.e. do the mitochondria become "coated" with BSA? If so, mitochondrial fraction protein would be overestimated and specific activities underestimated by the subtraction method. To resolve this point, proteins in such samples were separated by polyacrylamide gel electrophoresis. The amount of BSA in each sample was quantitated by comparison to BSA standards similarly prepared. Mitochondrial protein was then calculated by subtraction of the precisely measured BSA from total protein (Appendix 3).

Ultrapurification of Hepatic Mitochondria

Preparation of mitochondrial fractions by differential centrifugation of cell lysates leaves open the possibility that cosedimenting contaminants may be present in the final pellet. Of principal concern is the possible presence of peroxisomes, whose oxidative enzymes might interfere in measurement of mitochondrial oxygen uptake.

In control experiments, contaminants of mitochondrial fractions isolated by differential centrifugation were tested for by density gradient purification. Mitochondria were isolated on polyvinylpyrrollidone-coated silica-(Percoll^{TM}, Pharmacia [Blume 1979; Jenkins et al.

1979; Anonymous 1980])sucrose gradients. Three types of gradients were tested: 4 ml discontinuous step (1.05/1.10 g/ml), 4 ml continuous linear (1.07 - 1.15 g/ml), and 35 ml continuous self-generating sigmoidal (Blume 1979). Working gradient solutions were prepared by dilution of stock gradient material in grinding buffer and adjusted to neutral pH. Five ml cellulose-nitrate tubes were used in a Sorvall SW 50.1 swinging bucket rotor, and 37 ml polyallomer tubes were used in a Sorvall T865 fixed angle rotor, in a Sorvall OTD-2 ultracentrifuge. Four ml gradients were centrifuged for 40 min at 45,000 g (20,000 rpm), and 35 ml gradients for 45 min at 65,000 g (30,000 rpm), at 0 - 4 C. Large volume gradients were used most extensively. For checks of refractive index (density) profiles of the gradients (Figure 2), and for protein separation profiles, the bottoms of the tubes were pierced with an 18 gauge hypodermic needle and 4 - 5 drop fractions serially collected. Buoyant density profiles of control gradients were measured using a Bausch and Lomb Abbe-3L refractometer. Protein fractionation profiles were measured by the procedure of Bradford (1976). When desired, fractions were removed from the middle of a gradient by piercing the side of the tube and collecting the drops expressed by the weight of the gradient column on top.

The method of Guikema and Sherman (1980) for specific staining of peroxidase after separation of

mitochondrial fraction proteins by polyacrylamide gel electrophoresis was tried but rejected due to poor specificity in control experiments. The established kinetic pyrogallol method for peroxidase assay was satisfactory in control experiments with horseradish peroxidase as standard (Anonymous 1984).

Isolation of Coupled Lymphocyte Mitochondria

Difficulty of tissue sampling has been a major obstacle to mitochondria studies in some animal species. Peripheral lymphocytes are a potential sample source in such instances (Carlson and Kaneko 1973), as whole blood samples can be obtained simply and with minimum risk of injury to an animal. Leukocyte mitochondrial respiratory metabolism has been reviewed (Foster and Terry 1967; Kirschner, Getz and Evans 1972).

Peripheral lymphocytes were isolated from fresh bovine jugular vein blood samples using two different techniques. Blood was collected by veinipuncture in heparinized evacuated containers. Simultaneous separation of one fraction consisting of pure lymphocytes and another of polymorphonuclear leucocytes was achieved by gradient centrifugation of 10 ml whole blood, diluted 1:1 with 10 mM phosphate buffered saline (PBS) or RPMI 1640 culture medium. Twenty ml step gradients consisting of equal volumes of 1.077 g/ml and 1.199 g/ml ficoll (English and

Andersen 1974; Lucas et al. 1977; Ferrante and Thong 1980; Anonymous 1983) were prepared in siliconized 50 ml polycarbonate tubes. Loaded gradients were centrifuged for 40 min at 500 g (1600 rpm) using a JS4.2 swinging bucket rotor in a Beckman J-6B centrifuge at 25 C. Lymphocytes were collected by aspiration with a siliconized Pasteur pipette. This technique had the disadvantages of small sample capacity, tedious cell collection, and expense. A one step procedure utilizing prepackaged, self-forming silica sol gradients and a simple cell filter (Sepracell, Sepratech) was preferred.

Grinding with a serrated-tip teflon pestle in a 7 ml glass tissue homogenizer, or with a glass pestle in a 1 ml Dounce-type homogenizer, were found to be unsatisfactory for lymphocyte disruption, as the cells were resistant to mechanical lysis. Coupled mitochondria were finally successfully isolated by differential centrifugation of lymphocytes lysed by incubating in 25 U/ml heparin-PBS for 4.5 min, as described by Nessi et al. (1977). State 3 respiration of this preparation was very slow, 1.7 μM O_2/min from a 12 ml blood sample (approximately 2.4 x 10^6 lymphocyte cells) utilizing 250 μM ADP and 5 mM succinate. Respiratory control ratio was 3.40. The measured ADP:O ratio exceeded the theoretical maximum of 2.0. As alternate biopsy sources became available this procedure

was not further refined, though it may still be considered a viable alternative.

Studies Using Rabbit Hepatic Mitochondria

Methods of mitochondrial fraction isolation and respiratory metabolism measurement developed using mouse hepatic tissue were applied to samples of rabbit liver tissue. In two cases, mature female New Zealand rabbits were euthanized by cervical dislocation. Neither tissue samples transported to the laboratory in sucrose perfusion buffer before homogenization nor samples homogenized immediately after excision yielded coupled mitochondrial preparations. In a third case, a neonatal rabbit euthanized in the laboratory exhibited similarly uncoupled isolated mitochondria. Succinate was used as substrate. Mouse liver preparations run as controls, using identical lots of buffers, substrate and ADP, were well coupled. The reason that application of these procedures to rabbit liver samples was unsuccessful remains unknown, as this system was not further pursued.

Studies Using Chicken Hepatic Mitochondria

Applicability of mitochondrial isolation and measurement methods using chicken liver tissue samples was tested. White Leghorn eggs were hatched in a laboratory incubator, and day-old chicks euthanized by cervical dislocation. Hepatic mitochondrial fractions were isolated

by the general method outlined for mouse liver tissue. Mitochondria were well-coupled with succinate (RC ratio 3.24, ADP:O ratio 1.47) or pyruvate plus malate (RC ratio 2.24, ADP:O ratio 1.88) as substrate. However, no respiratory control was observed with alpha-ketoglutarate as substrate.

A study was conducted to test for differences in mitochondrial respiratory activities, and correlations of mitochondrial respiratory parameters with phenotypic traits of economic importance, in two genetically diverse poultry strains. Additionally, the activity of in vitro mixtures of mitochondria from the two strains was measured to test for possible interactive effects. Mature (11 - 12 mo) Hubbard White Mountain Cross broiler (live weight 4,117 $\pm$ 143 g) and White Leghorn layer (live weight 1,635 $\pm$ 65 g) hens were randomly selected from University of Arizona Poultry Research Center flocks. One bird of each strain was euthanized by cervical dislocation daily for nine d to obtain liver tissue samples for replicated mitochondrial assays. A uniform tissue sample (2.98 - 3.02 g) was obtained from each bird. Protein yield in the mitochondrial fraction per unit wet hepatic tissue was measured. A mitochondrial mixture was prepared by combining equal volumes of supernatant from the initial centrifugation of homogenates, representing equal wet tissue weights. After reservation of an aliquot for protein determination,

fatty acid-free BSA (5 mg/g wet tissue) was added to the final mitochondrial suspension.

Three respiration traces from separate aliquots of each mitochondrial preparation were recorded daily, with three respiratory cycles initiated with pulses of 83 μM ADP per trace. Succinate (2.5 mM) was utilized as substrate. The reaction mixture contained 1 ml of 3 mg mitochondrial fraction protein/ml suspension and 2 ml reaction buffer. Order was varied by strain, including the mixture, over days to compensate for possible preparation aging effects within each day.

Effects of breed and respiratory cycle on state 3 respiration rate, RC ratio and ADP:O ratio were analyzed statistically with a repeated measures analysis of variance (Hull and Nie 1981). The model included breed as the between subjects factor and cycle and trace (replication) as within subjects factors. Deviations of mixtures from their predicted values (means of the two strains) were tested by predetermined linear contrasts. Within breeds, rank correlations of mitochondrial yield and activity parameters with body weight and egg production rate (Leghorns only) were tested (Steel and Torrie 1980).

Studies Using Mouse Hepatic Mitochondria

A study was conducted to test for differences in mitochondrial respiratory capacity, and correlations of

mitochondrial respiratory parameters with phenotypic traits of economic importance in livestock species, among diverse genetically standardized mouse strains. A major goal of the study was comparison of mitochondria of hybrids heterotic for one or more traits with those of parental strains and of non-heterotic hybrids. Also, in vitro complementation of mitochondria from the corresponding inbred parental strains exhibiting differing degrees of combining ability was tested, to see if combining ability might be predictable from mitochondrial complementation.

Commercially available F_1 hybrid mice were poorly characterized with regard to phenotypic traits of economic importance in other species. The hybrids selected for the study were chosen on the single trait of postweaning growth rate, with uniform maternal effects. The BALB/cJ female x C57BL/6J male F_1 hybrid was positively heterotic for postweaning growth from age 3 to 9 wk, with body weights across sexes averaging 7.7% higher ($P<.01$) than midparent. The BALB/cJ female x A/J male F_1 hybrid was non-heterotic, with mean postweaning body weights to 9 wk not different ($P>.10$) than midparent (Heiniger and Dorey 1980). Marked phenotypic variation among the parental strains included the superior gain to feed consumption ratio and comparatively large mature body size of the A/J strain, and well-characterized reproductive superiority of the C57BL/6J strain. Balanced numbers of male and female mice, and all

possible combinations of male and female mixtures, were evaluated.

Hepatic mitochondrial fractions were isolated from mice 5 to 9 wk old by the general method outlined. Control mitochondrial preparations highly purified on density gradients indicated the differential centrifugation technique was satisfactory for comparative purposes. For complementation tests, aliquots of supernatant from the initial low-speed centrifugation step were mixed 1:1 on a wet tissue basis. After reservation of an aliquot for protein determination, fatty acid-free BSA (20 mg/g wet tissue) was added to the final mitochondrial suspension. The reaction mixture contained 0.5 ml of 1 mg mitochondrial fraction protein/ml suspension and 2.5 ml reaction buffer.

Four respiration traces from separate aliquots of each mitochondrial preparation, using two substrates, were recorded on each individual. Succinate (2.5 mM) and a mixture of pyruvate plus malate (2.5 mM + 2.5 mM) were used to test for differences in utilization of FAD- and NAD-linked substrate, respectively. Three respiratory cycles were initiated with pulses of 80 μM ADP per trace.

Respiratory data were analyzed using a repeated measures analysis of variance (Hull and Nie 1981; SAS Institute Inc. 1985) with strain and gender and their interaction effects tested separately for each substrate. Significance of differences among means was tested by

Fisher's protected LSD. Significance of complementation was tested by predetermined linear contrasts.

Pursuant to results of this study, androgen effects on mouse mitochondrial respiratory capacity were directly evaluated. In one trial, 57 d old C57BL/6J male mice were orchiectomized (Cunliffe-Beamer 1983) under ketamine-xylazine anesthesia, and their hepatic mitochondrial respiratory capacity compared to that of untreated control males 10 d later. In a second trial, 37 d old C57BL/6J female mice were treated with subcutaneous injections of 1 mg testosterone propionate every other day over 8 d (Koenig et al. 1980), and their hepatic mitochondrial respiratory capacity compared to that of control females injected with vehicle only and of untreated control males. Within trials all mice were littermates, and treatment groups were matched by initial body weight. Three mitochondrial respiratory cycles were measured on each of duplicate aliquots from each of three individuals per treatment group. Pyruvate (2.5 mM) plus 2.5 mM malate as substrate and 125 µM ADP were used. Differences between group means pooled over respiratory cycles were tested by predetermined t-tests.

Further characterization of F_1 hybrid mice with regard to reproductive performance and preweaning growth was undertaken. Matings were made to produce inbred, F_1 hybrid, F_2 selfed-hybrid, and three-way cross progeny.

Data were collected on progeny from each of four pairs of mice of each genotype combination. Inbred strains evaluated were A/J, C57BL/6J, BALB/cJ and DBA/2J. Four reciprocal F_1 hybrids between A/J and BALB/cJ, and C57BL/6J and BALB/cJ strains, were produced. These were selfed to produce four F_2 hybrid strains, and also crossed to the DBA/2J strain to produce eight three-way cross strains. Mouse husbandry was as described in Appendix 4. Traits recorded on each litter included live litter size, total litter size, litter and mean birth weight, weekly pre-weaning litter and mean weights for three wk, litter and mean weaning weight, weaning rate, and weekly post-weaning litter and mean weights for two wk. Strain effects were analyzed using least-squares general linear models procedures with birth date and litter size covariates. Specific comparisons were made with linear contrasts (SAS Institute Inc. 1985).

Studies Using Cow Hepatic Mitochondria

A study was conducted to test for differences in mitochondrial respiratory activities, and correlations of mitochondrial respiratory parameters with phenotypic traits of economic importance, in Holstein dairy cattle. Ten 2 - 4 yr old cows near the 70th d of current lactation were selected to include five with high (10,685 $\pm$ 404 kg) and five with low (8,221 $\pm$ 273 kg) mean 305 d adjusted milk

yields. Also, five 2 - 4 yr old non-lactating cows near the 8th mo of gestation, chosen randomly with respect to milk yields, were included. Cows were obtained from the University of Arizona Dairy Research Center.

Liver tissue samples were obtained by percutaneous biopsy (Smart and Northcote 1985). Preliminary trials had demonstrated coupled mitochondria could not be isolated from liver samples obtained during the normal slaughter process, whether transported to the laboratory in sucrose perfusion buffer or homogenized immediately after excision. This was probably due to the 20 - 30 min period of ischemia before samples could be obtained. Cattle were restrained only with a headgate. After local anesthesia was achieved by subcutaneous infiltration of 5 cc of 2% lidocaine HCl, a 1 cm vertical incision was made, using a #11 scalpel blade, between the 11th and 12th ribs, approximately 45 cm ventrad from the dorsal midline. Tissue cores were obtained using a 15 cm x 2 mm biopsy needle (Tru-Cut, Travenol). Approximately 10 - 15 cores, 0.5 - 1.5 cm x 2 mm, were obtained from each animal. Tissue samples were immediately homogenized using a serrated-tip teflon pestle in a 7 ml hand tissue grinder, or a glass pestle in a 7 ml Dounce-type homogenizer, in 0.5% BSA-containing buffer, and held on ice while transported to the laboratory. Skin incisions were closed with stainless-steel staples and covered with

spray-on antiseptic plastic bandage. The biopsy had no effect on subsequent lactation performance.

Mitochondrial fractions were isolated by differential centrifugation. Respiratory measurements were made using 0.375 ml aliquots of 2.3 mg mitochondrial fraction protein/ml suspension and 1.125 ml reaction buffer. Succinate (6 mM) and a mixture of pyruvate (6 mM) plus malate (6 mM) were tested as substrates. Respiratory cycles were initiated with pulses of 80 µM ADP.

Consistently poor mitochondrial samples were obtained from gestating cows, perhaps due to increased blood flow to the liver at the late stage of gestation. Consequently these samples were excluded from statistical analyses. No respiratory control was exhibited with pyruvate plus malate as substrates. A variable number of mitochondrial respiratory cycles was measurable on one or two aliquots prepared from each sample. First cycle state 3 respiration rate, RC ratio and ADP:O ratio linear and rank correlations with sire progeny predicted difference, dam progeny index, cows own index, and mean 305 d adjusted yields for milk and milk fat were calculated. Significance of correlations was tested with the appropriate F or t statistic.

In a second study, differences in mitochondrial respiratory capacity among Angus, Brangus, and Hereford cows, and within-breed correlations of mitochondrial

respiratory parameters with phenotypic traits of economic importance, were tested. Ten 5 - 8 yr old Angus cows in the 5 - 10th wk of lactation, ten 3 - 13 yr old Brangus cows in the 4 - 12th wk of lactation, and ten 3 - 8 yr old Hereford cows in the 6 - 10th wk of lactation, obtained from University of Arizona herds, were sampled by percutaneous hepatic biopsy as outlined. Cattle were selected to represent extremes within breed on the basis of calculated maternal growth breeding value. Also, four Angus, three Brangus and three Hereford non-lactating cows were sampled. Cattle were restrained in a squeeze chute. The liver could not be located on two nulliparous Brangus cows (interestingly these were slightly inbred half-sibs). A hepatic abcess was biopsied on one Brangus cow. Two Brangus cows were too intractible for sampling.

Uniform amounts of tissue (approximately 0.25 g) were obtained from each animal. Mitochondrial samples were isolated by differential centrifugation. Respiratory measurements were made using duplicate 0.375 ml aliquots of 3.9 mg mitochondrial fraction protein/ml suspension and 1.125 ml reaction buffer. Twelve unreplicated complementation mixtures of Angus and Hereford mitochondria were prepared, in proportions unknown prior to subsequent protein assay of donor suspensions. Succinate (6 mM) was used as substrate. Three respiratory cycles were initiated with successive pulses of 80 μM ADP per trace.

Respiratory data were analyzed using a repeated measures analysis of variance (SAS Institute Inc. 1985) with breed as the between subjects factor and cycle and trace (replication) as within subjects factors. Cow age and day of lactation were covariates in least-squares linear stepwise models of weaning and yearling growth traits of cows, their maternal and paternal half-sibs and progeny regressed on mitochondrial respiratory parameters. Significance of mitochondrial effects was assessed by testing standardized regression coefficients. Significance of complementation was tested by chi-square analysis, with predicted means calculated as outlined in Figure 6.

RESULTS AND DISCUSSION

This section begins with results of control experiments useful for interpretation of the series of comparative trials conducted. Results of studies on different species are described and discussed in turn.

Conditions During Respiration and Oxidative Phosphorylation

Conditions during in vitro measurements of mitochondrial respiratory metabolism must be completely described to permit critical comparison. As factors such as temperature and reaction buffer composition tend to be uniform within a study, and reducing substrate is supplied in excess of limiting amounts, the quantity of mitochondria in a reaction and the amount of ADP supplied tend to vary the most. Before comparative studies were begun, effects of these factors on measured respiratory parameters were assessed in control experiments.

Table 3 shows the effect of varying the quantity of mitochondria in the reaction. As intuitively expected, there is a direct relationship between rate of oxygen uptake and quantity of mitochondria. At very low concentrations of mitochondria (less than approximately 50 µg mitochondrial protein/ml reaction) the oxygen electrode is not sensitive enough to measure steady states with

Table 3. Effects of quantity of mitochondria on respiratory parameters.

Relative Mitochondrial Concentration (%)	State 3 (%)	State 4 (%)	RC Ratio (%)	ADP:O Ratio (%)
100[a]	100	100	100	100
83	89	83	101	103
67	72	66	102	98
50	53	47	107	107
33	37	33	98	96
17	---	---	---	---
Linear Correlation	.997**	.999**	$-.090^{ns}$	$.146^{ns}$

[a]Approximately 0.2 mg protein/ml, aliquoted for dilutions. Data are means of duplicates of mouse hepatic mitochondria utilizing succinate.

**P<.01

$^{ns}P>.10$

precision. Therefore it is crucial that mitochondrial concentration be precisely quantitated for comparative purposes. Any factor, unaccounted for, causing differences in mitochondrial quantity between different reactions can lead to erroneous comparisons. However, there is no relationship between quantity of mitochondria in the reaction and measured respiratory control or ADP:O ratios over this range. Each respiratory parameter thus seems to be a measure of the metabolism of individual mitochondria, showing no colligative properties.

The accepted K_m for ADP in mammalian oxidative ATP synthesis is 20-25 μM (Jacobus, Moreadith and Vandegaar 1982; Chance et al. 1986). Varying the ADP concentration can affect the complex stoichiometry of mitochondrial oxidative phosphorylation, reflected in respiration rate (Table 4). Measurements made using 20 μM ADP should be the most informative with regard to normal metabolism. However under practical in vitro conditions the trace deflection caused by a pulse of ADP to 20 μM is so slight as to be only ambiguously measurable. Above this concentration the amount of ADP added is arbitrary, in practice limited by solubility of crystalline ADP. Increasing exogenous ADP concentration also apparently tended to increase the ADP:O ratio. This may be explained by assuming that respiration uncoupled from oxidative phosphorylation is to some degree additive during state 3 respiration. It would constitute a

Table 4. Effects of ADP concentration on respiratory parameters.

ADP	Succinate rel.[a] 3	Succinate rel.[a] 4	Succinate RC[b]	Succinate PO[c]	Alphaketoglutarate rel.[a] 3	Alphaketoglutarate rel.[a] 4	Alphaketoglutarate RC[b]	Alphaketoglutarate PO[c]	Pyruvate rel.[a] 3	Pyruvate rel.[a] 4	Pyruvate RC[b]	Pyruvate PO[c]
Trial 1												
10 µM	32	78	1.68	1.13	---	---	----	----	100	100	1.79	1.67
30 µM	43	66	3.08	1.46	---	---	----	----	112	96	2.09	1.78
Trial 2												
60 µM	100	100	4.32	0.64	100	100	2.85	1.43	---	---	----	----
120 µM	102	105	4.22	1.02	114	115	2.80	1.28	---	---	----	----
180 µM	107	109	4.25	1.43	---	---	----	----	---	---	----	----
240 µM	---	---	----	----	108	92	3.33	2.00	---	---	----	----
360 µM	136	132	4.45	1.71	---	115	3.80	2.34	---	---	----	----

[a]Percent of arbitrary baseline (100) for States 3 and 4 respiration rate.

[b]Respiratory control ratio.

[c]ADP:O ratio.

comparatively smaller proportion of total oxygen uptake when state 3 is increased due to increasing ADP, resulting in an apparent increase in efficiency of oxygen utilization. These results demonstrate accurate ADP quantitation is also crucial to avoid erroneous comparisons. There was no marked effect of ADP concentration on respiratory control except at extremes. It was further considered that the cumulative amount of ADP added over multiple cycles within a trace, affording an opportunity for ATP concentration to build, might affect respiratory parameters. The data of Table 5 suggest such an effect might be important, especially with alpha-ketoglutarate as substrate, if comparisons are not exactly the same (for example, if more cycles are consistently obtained from a given mitochondrial source).

Results of inhibitor screening are presented in Table 6. These data serve chiefly to confirm the mitochondrial preparations responded as expected, and, for the appropriate substrate-inhibitor combinations, that utilization of other endogenous substrates did not contribute substantially to observed respiration.

Results of Gradient Purification

Figure 2 illustrates a representative density gradient profile with a superimposed protein fractionation profile. These results confirm the differential

Table 5. Effects of cumulative amount of ADP added[a].

Substrate	ADP Added	Previous Total	---Relative %---- State 3	State 4	RC Ratio	AD :0 Ratio
Succinate						
	60 µM	0	100	100	4.32	0.64
	60 µM	300 µM	68	---	----	----
	120 µM	0	100	100	4.22	1.02
	120 µM	60 µM	101	96	4.45	1.20
	120 µM	180 µM	88	100	3.70	1.50
	180 µM	0	100	100	4.25	1.43
	180 µM	180 µM	94	---	----	----
Alpha-keto-glutarate						
	60 µM	0	100	100	2.85	1.43
	60 µM	540 µM	124	92	3.83	2.00
	60 µM	600 µM	127	92	3.92	1.88
	120 µM	0	100	100	2.80	1.28
	120 µM	60 µM	119	136	4.55	2.00
	120 µM	420 µM	131	87	4.23	2.22
	120 µM	600 µM	148	87	4.77	2.14
	240 µM	0	100	100	3.33	2.00
	240 µM	120 µM	138	117	3.93	2.00
	240 µM	360 µM	173	108	5.31	2.45
	360 µM	0	100	3.80	2.34	
	360 µM	360 µM	102	---	----	----

[a]Change in mitochondrial activity relative to initial conditions when previous total = 0.

Table 6. Effects of inhibitors on mitochondrial respiration[a].

Inhibitor	Concentration	Substrate	State 4% of State 3
Rotenone	0	Succinate	20.8
Rotenone	10 mM	Succinate	20.3
Rotenone	0	Betahydroxybutyrate	28.0
Rotenone	10 mM	Betahydroxybutyrate	100
Malonate	0	Succinate	23.3
Malonate	6 mM	Succinate	12.3
Propyl gallate	0	Pyruvate	42.3
Propyl gallate	1 mM	Pyruvate	40.7
Propyl gallate	2 mM	Pyruvate	22.8
Propyl gallate	3 mM	Pyruvate	9.8
Propyl gallate	4 mM	Pyruvate	4.9
Propyl gallate	5 mM	Pyruvate	3.3
Antimycin a	0	Pyruvate	41.9
Antimycin a	0.2 µM	Pyruvate	18.6
Antimycin a	0.4 µM	Pyruvate	17.7
Antimycin a + 1 mM Propyl gallate	0.4 µM	Pyruvate	14.5
Antimycin a + 2 mM Propyl gallate	0.4 µM	Pyruvate	15.3

[a]Solvent ethanol had no effect.

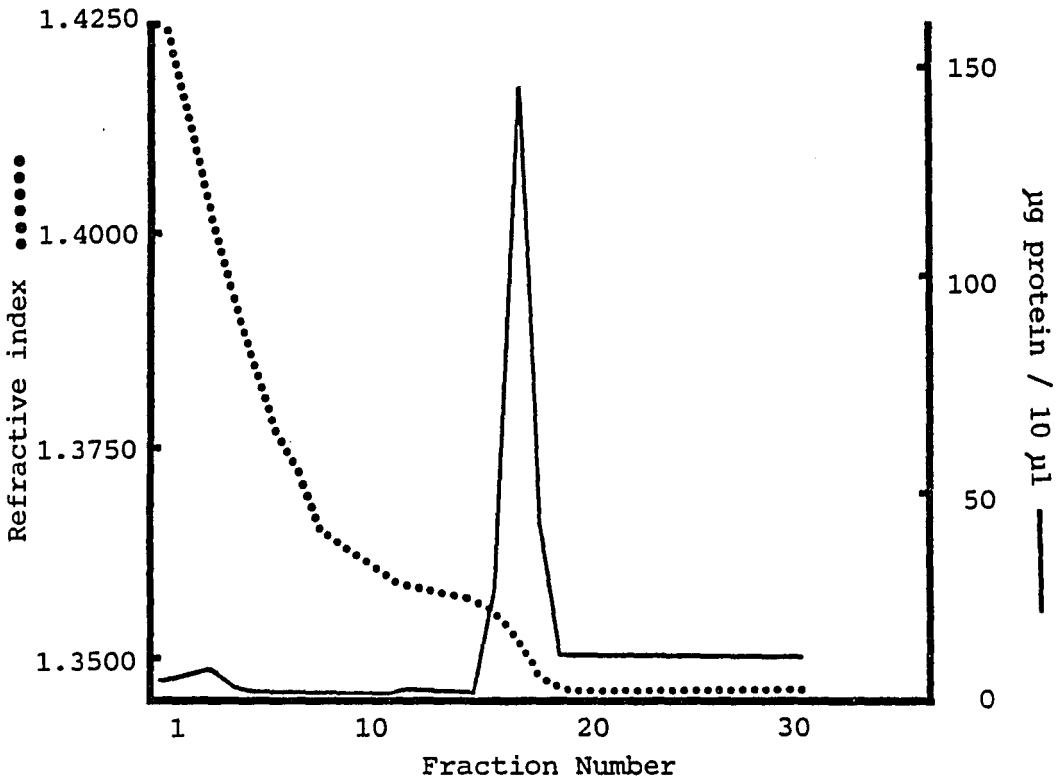

Figure 2. Protein fractionation profile of gradient purified mitochondria.

centrifugation protocol cleanly isolated the fraction of hepatic subcellular components of the narrow buoyant density range from 1.05 to 1.10 g/ml which includes mitochondria. Resolution of mitochondrial bands at their expected densities was reliably repeatable. Especially important is the absence of a band in the 1.13 g/ml density range which would include interfering microsomes. The uniform protein level present in the top of the gradient is probably soluble BSA (see also Appendix 3). Differential centrifugation according to the described protocol is appropriate for comparative mitochondrial studies.

Respiration measurements were made on gradient-purified mouse liver mitochondria. Gradient purification had adverse effects on mitochondrial quality. Respiration rates decreased markedly after purification by density gradient centrifugation. Respiratory control ratios decreased by nearly 50 percent. Gradient purification may be warranted for specific enzyme activity assays; these results point out it is not indicated for respiratory measurements on intact animal mitochondria. Collot, Wattiaux-DeConinck and Wattiaux (1975) studied mammalian mitochondrial deterioration under such conditions, and concluded it may be due to increased sucrose permeability under high hydrostatic pressure. Interestingly, plant mitochondria apparently resist deterioration under identical conditions.

Hepatic Mitochondrial Respiratory Activity of Chickens

Results of chicken hepatic mitochondrial respiratory activity measurements are presented in Table 7. State 4 respiration rates of Leghorn mitochondria were 6-7 times lower than previously reported for succinate (Dziewiecki and Kolotaj 1976, 1980), possibly because mitochondria of the current study were much more tightly coupled. State 3 respiration rate increased ($P<.05$) from the first through the third cycles, though state 4 rate was constant across cycles. The efficiency of phosphorylation tended to be higher in the second cycle. Differences between strains did not reach statistical significance, though broilers consistently ranked higher across cycles for each mitochondrial respiratory trait measured. Consistently more ($P<.05$) protein was isolated in the mitochondrial fraction per unit wet tissue weight from broiler hens ($7.8 \pm .2$ mg/g) than layer hens ($6.2 \pm .2$ mg/g), strongly suggesting the broiler hens had more mitochondria per unit wet tissue. Based on these yields and on calculated ATP synthesis rates (Table 7), and assuming that liver weight adjusted for body weight did not differ between breeds (Brown, Southern and Baker 1985), the capacity for hepatic oxidative phosphorylation was 38.5% higher in broilers than in layers (277 vs. 200 μM ATP/ g liver/min). Consistent with other studies, the heavier body weight strain tended to have more rapid

Table 7. Respiratory activities of individual and mixed mitochondrial isolates from two strains of chickens[a].

Strain	$\mu M\ O_2$/mg prot/min State 3	$\mu M\ O_2$/mg prot/min State 4	Respiratory Control Ratio	ADP:O Ratio (μM ADP/μA O)	ATP Synthesis (μM/mg prot/min)
Hubbard	16.63	4.62	3.68	1.06	35.45
Leghorn	15.60	4.64	3.34	1.03	32.24
1:1 Mixture	16.03	4.69	3.44	1.03	-----
Pooled SEM	0.41	0.08	0.01	0.01	2.97

[a]Data are means for nine hens per strain, pooled over three respiratory cycles and three replicates (replication effect $P<.10$) per individual (all interaction effects $P>.10$). No strain difference reached statistical significance. No mixture differed ($P>.10$) from predicted values tested by predetermined linear contrasts.

mitochondrial respiration. This is in agreement with the finding of Kuenzel and Kuenzel (1976) that, expressed as kcal/bird, broilers have higher basal metabolic rates. However, expressed on a per unit total body weight (p. 38) basis, the ranking reverses (.1223 for Leghorns vs. .0672 for broilers), again paralleling the finding for comparative adjusted basal metabolic rates.

By adjusting to a unit body weight basis, previous interpretations of basal metabolic rate have implicitly assumed the animal body to be homogeneous with respect to oxygen consumption. Some tissues are clearly more energetically active than others, and body composition may also be quite variable. Therefore subcellular metabolic differences, such as a difference in oxidative ATP synthesis capacity, which may directly underly between-breed variation in body weight, can be easily masked by such adjustments if body weight differences are large.

No significant within-breed rank correlation, or trend toward association of any mitochondrial parameter with body size was found (Table 8). Sample size was probably too small, and within-breed body-weight ranges too limited, for any such tendency to be detected. The same was true for egg production rate, for which phenotypic variation was very limited (0.79 $\pm$ 0.02 eggs/d over a three mo period). Based on these results, effects of possible within-breed variation in mitochondrial activity on these

Table 8. Rank correlations of mitochondrial traits with performance traits within breeds of chickens[a].

Trait	State 3 Rate	Respiratory Control Ratio	ADP:O Ratio	Mitochondrial Yield
Body Weight				
Broilers	.121	.200	.050	.004
Layers	-.150	.050	.150	.554
Eggs/day				
Layers	.167	.500	.450	-.088

[a]Correlations are calculated from nine individuals per strain. Minimum significant (alpha=.10) r=.582.

traits appear to be minor in comparison to other causes of variation. The magnitude of possible mitochondrial effects on production traits may be better estimated from designed experiments with large numbers of animals with large ranges in phenotypic variation.

No deviation from the predicted means of inbred sources were detected for any respiratory trait of mixed mitochondria (Table 7). These therefore constituted further "consistent negative" results since, as Leghorn X broiler reciprocal hybrids are not commonly heterotic for growth or egg production rates (Nordskog and Phillips 1960), mitochondrial complementation would not be expected. Further studies using different substrates and younger birds of breeds with defined combining ability would be necessary for a more complete evaluation of mitochondrial complementation in chickens.

Respiratory Activity and Complementation of Mouse Hepatic Mitochondria

Table 9 presents results of respiratory measurements of hepatic mitochondria of inbred and hybrid mice. Significant differences in state 3 respiration rate, respiratory control ratio and ADP:O ratio were detected among inbred strains. Mitochondria of the C57BL/6J strain had higher ($P<.05$) respiratory control ratio, and females of this strain higher state 3 respiration rates (strain by

Table 9. Strain and gender effects on respiratory metabolism of mouse hepatic mitochondria[a].

Strain	Sex	n	State 3 Respiration (μM O_2/mg prot/min)		Respiratory Control Ratio (State 3/State 4)		ADP:O Ratio (μM ADP/μA O)	
			Succinate	Pyruvate+ Malate	Succinate	Pyruvate+ Malate	Succinate	Pyruvate+ Malate
A/J	M	10	26.0	5.9	3.64	2.43	1.13	1.47
	F	10	24.9	5.9	3.83	2.72	1.18	1.61
C57BL/6J	M	10	25.4	4.6	4.08	2.30	5.16	1.38
	F	10	32.3	7.8	4.04	2.78	1.17	1.53
BALB/cJ	M	20	25.2	6.2	3.75	2.77	1.15	1.54
	F	20	26.5	7.1	3.84	2.88	1.15	1.61
BALB/cJ X C57BL/6J	M	5	29.4	6.4	3.97	2.59	1.09	1.52
	F	5	28.9	6.9	4.10	2.86	1.17	1.52
BALB/cJ X A/J	M	5	27.5	6.7	3.65	2.52	1.17	1.54
	F	5	25.7	6.7	3.82	2.97	1.15	1.69
Pooled SEM			0.84	0.24	0.09	0.09	0.03	0.04

[a]Strain and sex effects $P<.02$, except succinate respiratory control ratio control ratio $P<.10$ and ADP/O n.s., in repeated measures analyses of variance separate for each substrate. Mice were 5 to 9 weeks old. Succinate = 2.5 mM succinate, pyruvate + malate = 2.5 mM pyruvate + 2.5 mM malate.

sex interaction $P<.01$), utilizing FAD-linked succinate, than those of other inbred strains. Mitochondria of this strain have also been shown to have the highest rate of protein synthesis of strains compared (Wagner 1972). No pattern of variation in state 4 respiration was detected. Interstrain differences in respiration rates and respiratory control ratios did not reach statistical significance with NAD-linked pyruvate plus malate as substrates, though the tendency for rapid state 3 respiration by mitochondria of female C57BL/6J mice was pronounced. Efficiencies of oxidative phosphorylation (ADP:O ratios), titrated with 80 μM ADP, were 50 - 60 percent of theoretical maximums across all strains. With NAD-linked substrate, mitochondrial phosphorylation of BALB/cJ mice was more efficient ($P<.05$) than that of C57BL/6J mice, with the A/J strain intermediate.

Calculations based on the data in Table 9 demonstrated that, with the exception of C57BL/6J females, inbreds had remarkably uniform ATP synthesis rates utilizing succinate (approximately 59 ± 4 μM ADP esterified/mg protein/min). The C57BL/6J females' mitochondria were estimated to synthesize ATP at a 28% faster rate than other inbreds utilizing succinate. This advantage increased to 30 percent for mitochondria utilizing pyruvate plus malate (male C57BL/6J mouse mitochondria had markedly low ATP synthesis rates with this substrate). Calculated ATP

synthesis rates of hybrids were uniform and not different from inbreds. As in inbreds, males with C57BL/6J dams had markedly lower ATP synthesis rates utilizing pyruvate plus malate than did other genotypes. Across strains, ATP synthesis rates of hepatic mitochondria of mice were about twice those of hepatic mitochondria of chickens under similar conditions.

Based on comparisons with data from the literature (Heiniger and Dorey 1980), the high respiration rates, respiratory control ratios and ATP synthesis rates of female C57BL/6J mice can be associated in a general way with their well-characterized superiority for reproductive traits, such as percent productive matings, percent having a minimum of four litters, age at first litter, and mean number born per litter. The comparatively large mature body size of A/J mice apparently had no tendency for association with any mitochondrial trait.

No pattern of significant mitochondrial heterosis for respiration rates, respiratory control ratios or ADP:O ratios was observed for any hybrid. In the only comparable study published, Chai and Mukherjee (1974) were able to distinguish one mouse hybrid with superior state 1 respiration. Efficiency of phosphorylation of CAF1 and CB6F1 hybrids was not different from maternal BALB/cJ inbreds.

The C57BL/6J inbred strain had consistently lower ($P<.01$) mitochondrial protein yield per unit wet hepatic tissue than A/J and BALB/cJ inbred strains (12.15 $\pm$ 0.54 vs. 15.20 $\pm$ 0.54 and 14.23 $\pm$ 0.38 mg/g wet tissue, respectively, suggesting indirectly that the C57BL/6J mice had fewer mitochondria per unit wet tissue. Male inbred mice consistently tended to yield more mitochondrial protein per unit wet tissue than did female inbreds. However, the reverse was consistently true for hybrids. A highly significant strain by sex interaction was observed for mitochondrial protein yield. Also, hybrids as a group yielded more (18.64 $\pm$ 0.77 mg/g, $P<.01$) protein in the mitochondrial fraction than did inbreds. Increased numbers of mitochondria in hybrid animals compared to inbreds have been detected previously by electron microscopy (Murzamadiev 1970). The implied increased mitochondrial concentration appeared to be of equal magnitude for each hybrid genotype. Based on these yields, and data of Table 9, hybrids had greater total hepatic energy synthesis capacity than did inbreds (1185 vs. 848 $\pm$ approximately 50 μM ATP/g liver/min). At age 5 wk, C57BL/6J mice had significantly lower body weights than A/J and BALB/cJ strains (Heiniger and Dorey 1980). Males of all strains had higher body weights than females. Hybridization thus reversed the tendency, apparent from both chicken and mouse

data, for mitochondrial yield per unit wet hepatic tissue to be somewhat dependent on body size.

Original data on preweaning and postweaning growth of CAF_1 and $CB6F_1$ hybrids and their parents are summarized in Figure 3. Though body weight deviations from midparent did not reach statistical significance for either hybrid, the $CB6F_1$ was consistently heavier, and the CAF_1 consistently lighter than midparent until weaning. Beyond weaning, results were very similar to literature data, confirming that the $CB6F_1$ was significantly heterotic for postweaning growth while the CAF_1 did not differ from midparent. Also, with the same maternal genetic prenatal environment, the live birth rate of $CB6F_1$ hybrids was higher than that of CAF_1 hybrids (0.999 vs. 0.872 ± 0.046, $P<.06$), further evidence of differences in vigor between the hybrids. There were no differences between hybrids in growth of their F_2 or three-way cross progeny (Figures 4 and 5).

When mitochondria of C57BL/6J and BALB/cJ females (the strains chosen because their hybrid, $CB6F_1$ mice, were heterotic for postweaning growth) were mixed in vitro, enhanced state 3 respiration exceeding that predicted, and of the more rapidly respiring strain, was observed with succinate ($P<.10$) and pyruvate plus malate ($P<.05$). No complementation was observed for state 4 respiration rates. Also, respiratory control ratio of these mixtures utilizing

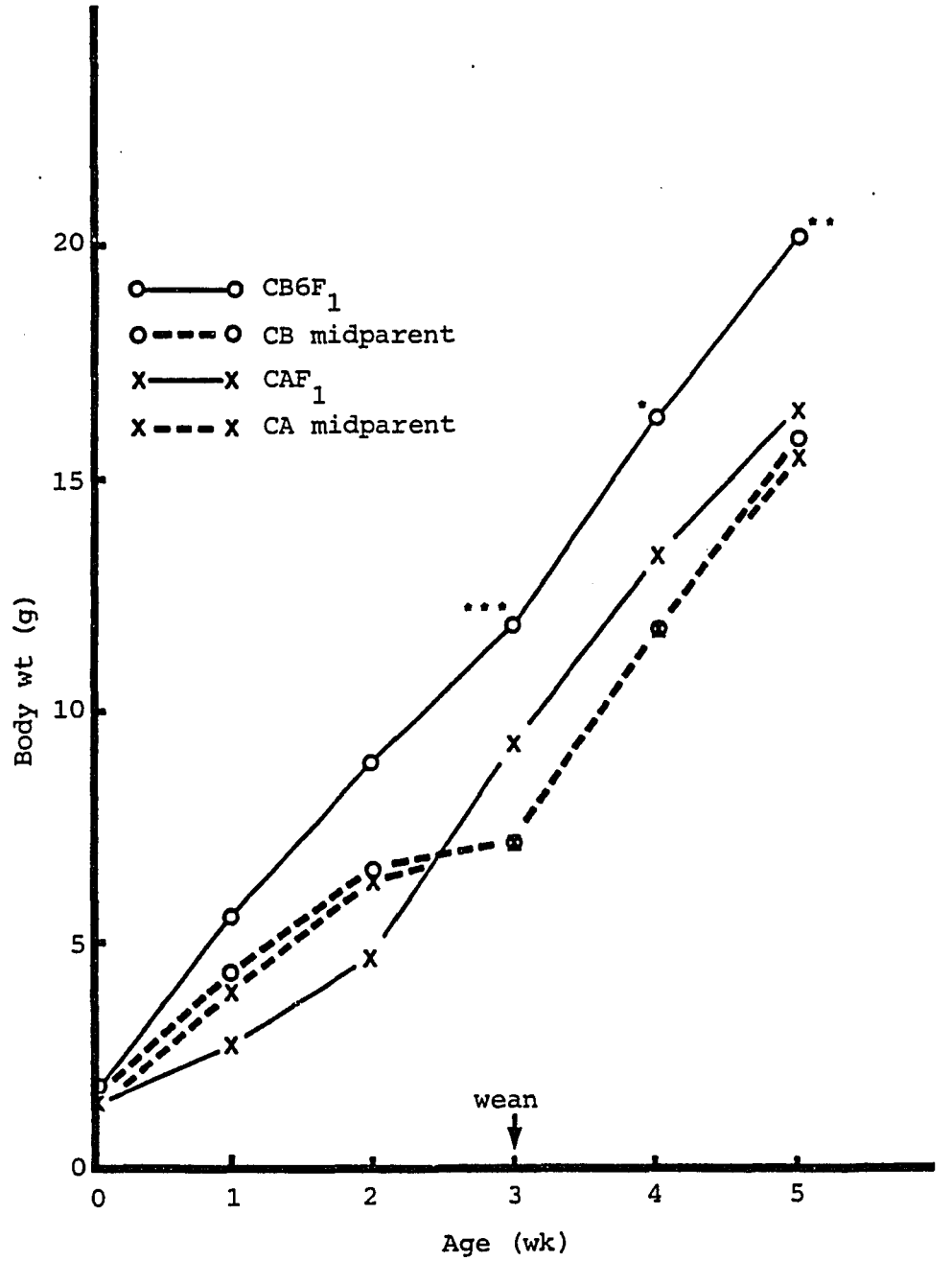

Figure 3. Body weight changes of hybrid mice and parents. Significance of difference from midparent is indicated (*=P<.05, **=P<.01, ***=P<.001).

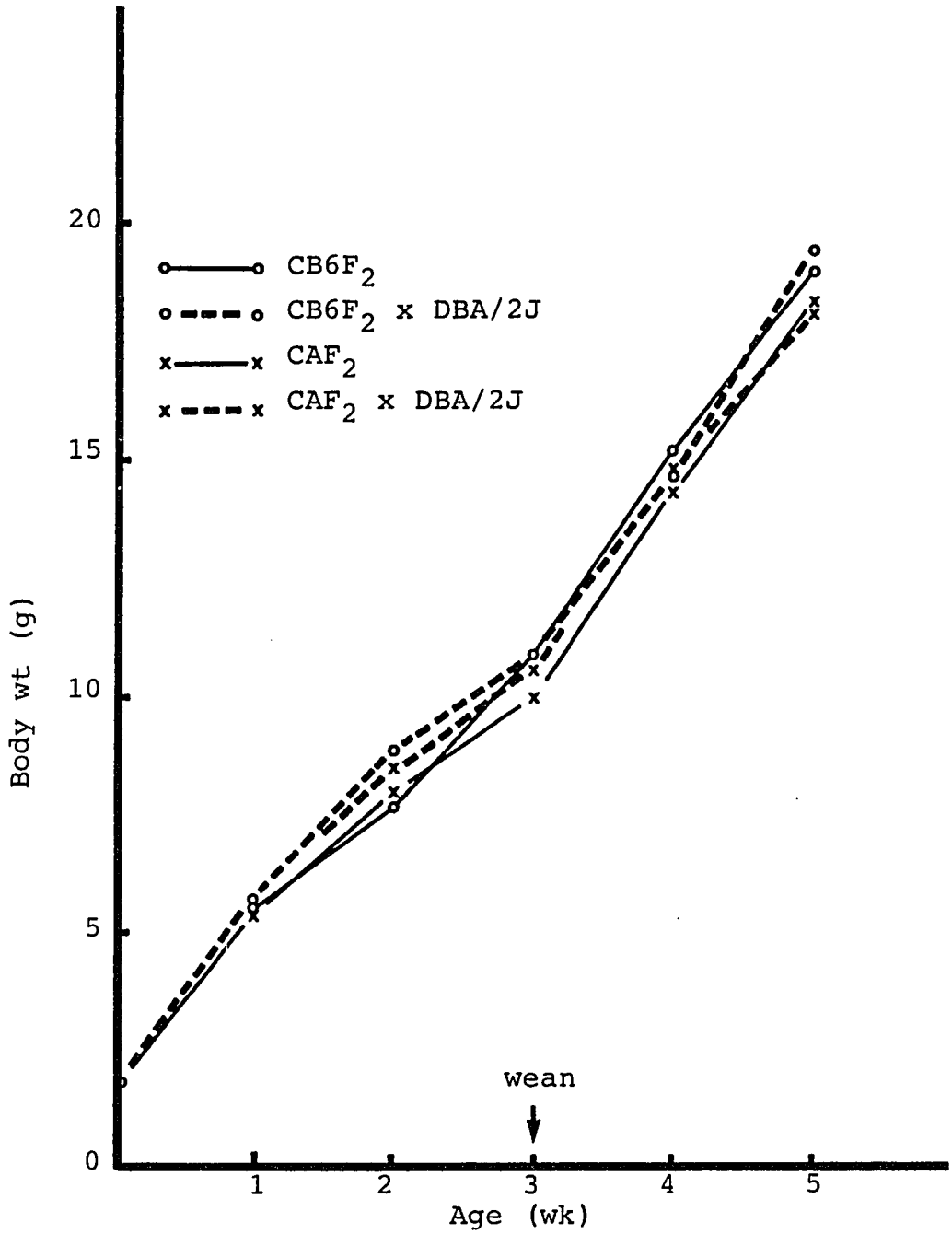

Figure 4. Body weight changes of progeny of hybrid female mice. Differences between hybrids were not significant.

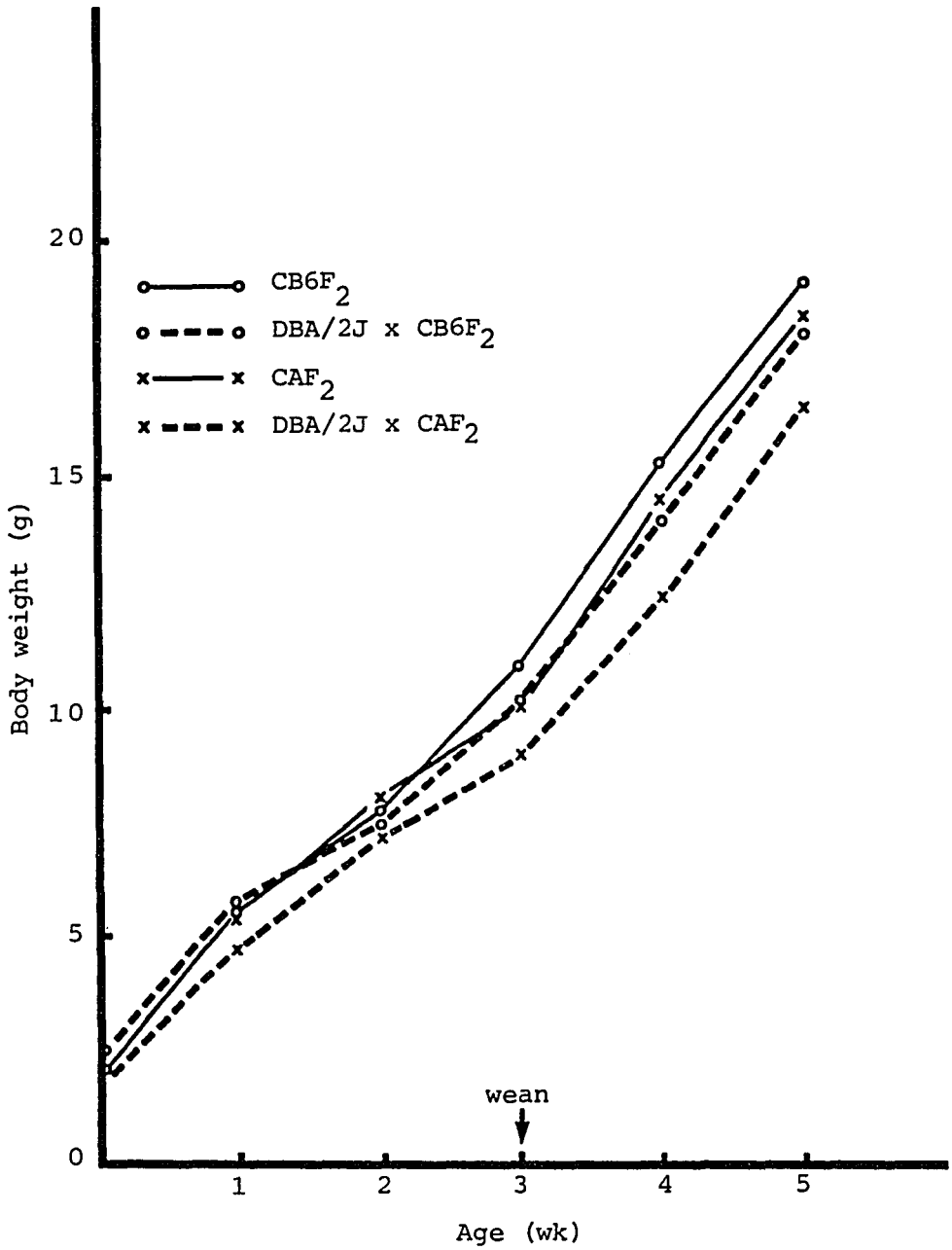

Figure 5. Body weight changes of progeny of hybrid male mice. Differences between hybrids were not significant.

succinate was higher ($P<.05$) than that predicted, and higher than the more tightly coupled strain. No significant tendency toward complementation was detected for ADP:O ratios (Table 10).

No complementation for any mitochondrial respiratory parameter was detected under any conditions for mixtures of mitochondria of A/J and BALB/cJ mice (Table 10). These mixtures, made as controls (CAF_1 mice were non-heterotic for any trait), indicated there were no unaccounted for effects which might have consistently induced spurious results.

Potential pitfalls in interpretation of data from mixing experiments were recognized. Figure 6 shows how unaccounted for deviations from a true 1:1 mixture (the most commonly used ratio in mitochondrial mixing experiments, though not the only ratio previously tested [McDaniel and Sarkissian 1970; French 1971]) can lead to erroneous predictions regarding respiration rates of mixtures. Error arises when variation in mitochondrial concentration among samples is not considered. The effect is magnified with large differences in respiration rate between sources, a condition perhaps most likely to exist when sources are genetically diverse (i.e. when complementation has been most likely to be observed). Due to this uncertainty, unless the contribution of each source to the mixture was precisely quantitated, only respiration rates

Table 10. Strain and gender effects on complementation of mouse hepatic mitochondria[a].

Mitochondrial Sources		State 3 Respiration (μM O_2/mg prot/min)		Respiratory Control Ratio		ADP:O Ratio (μM ADP/μA O)	
			Pyruvate		Pyruvate		Pyruvate
A/J	BALB/cJ	Succinate	Malate	Succinate	Malate	Succinate	Malate
M	M	25.04	5.79	3.77	2.74	1.19	1.61
M	F	24.46	5.92	3.82	2.47	1.13	1.46
F	M	23.94	5.65	3.92	2.69	1.17	1.56
F	F	25.67	6.44	3.75	3.09	1.18	1.68
C57BL/6J	BALB/cJ						
M	M	25.12	5.32	3.91	2.48	1.19	1.46
M	F	25.10	5.53	3.81	2.47	1.11	1.47
F	M	28.77	7.33	3.76	2.62	1.12	1.45
F	F	34.06	8.95	4.40	3.02	1.22	1.61
Pooled SEM		1.31	0.36	0.16	0.13	0.02	0.04

[a]Data are means of five replicates each. Complementation was assessed by comparison to appropriate pooled means for each strain and sex combination (Table 9).

Sample "A"	Sample "B"
Concentration = 8 mg/ml	4 mg/ml
Respiration = 20 units/mg	16 units/mg
Respiration/ml = 160 units	64 units

- 1 ml:1 ml mixture = 12 mg/2 ml = 6 mg/ml
- true predicted repiration/ml = [8/12)*20 + (4/12)*16]*6
  = 112 units
- erroneous predicted respiration/ml = [20 + 16]/2]*6
  = 108 units
- apparent enhancement = (112 - 108)/108 = 3.7%

Figure 6. Example of incorrectly predicted respiration rate of a mitochondrial mixture.

of mixtures statistically exceeding the more rapidly respiring source reliably indicate some interaction. Enzyme activities of mixtures may be susceptible to erroneous predictions in the same manner. Figure 7 shows how inappropriate predictions of respiratory control ratios for mixtures can lead to incorrect conclusions regarding this trait. The correct predicted state 3 rate for a true 1:1 mixture is the mean state 3 rate of the two sources assayed separately, and the same is true for the correct predicted mean state 4 rate. The correct predicted respiratory control ratio of a mixture is therefore the quotient of the mean state 3 and mean state 4 rates, not simply the mean of the respiratory control ratios of the two sources assayed separately. Again, unless the method for calculating the predicted respiratory control ratio of mixtures was carefully described, only mixtures with respiratory control statistically exceeding that of the more tightly coupled source are reliable. Figure 8 shows that, for fixed ADP concentration, the relationship between oxygen uptake and ADP:O ratio is non-linear. The ADP:O ratio of a mixture cannot be predicted as the simple mean of the two sources assayed separately. It can be correctly predicted only from the mean amounts of oxygen consumed during phosphorylation (previous examples of patently incorrect predictions include those of Ellis, Brunton and Palmer [1972] and Barratt and Flavell [1977]). As for

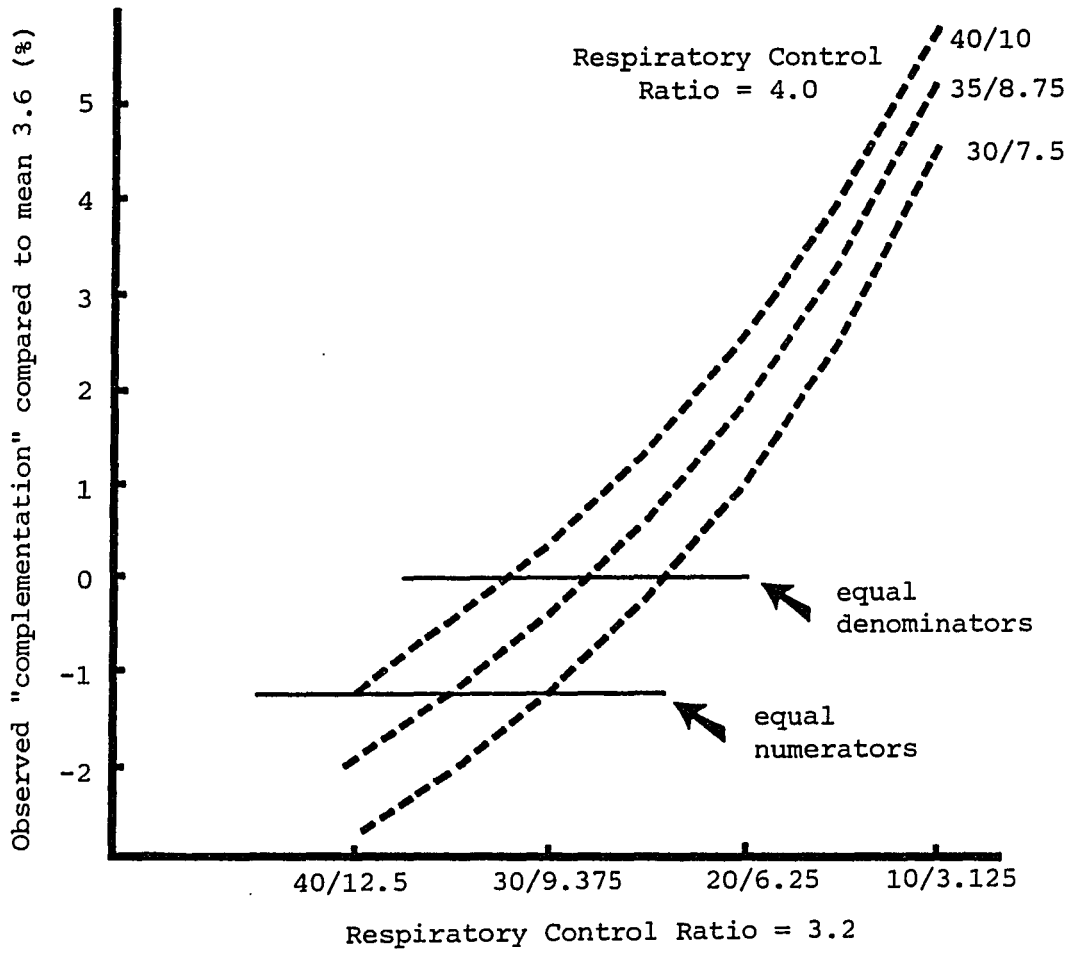

Figure 7. Apparent effect of incorrectly predicted respiratory control ratio.

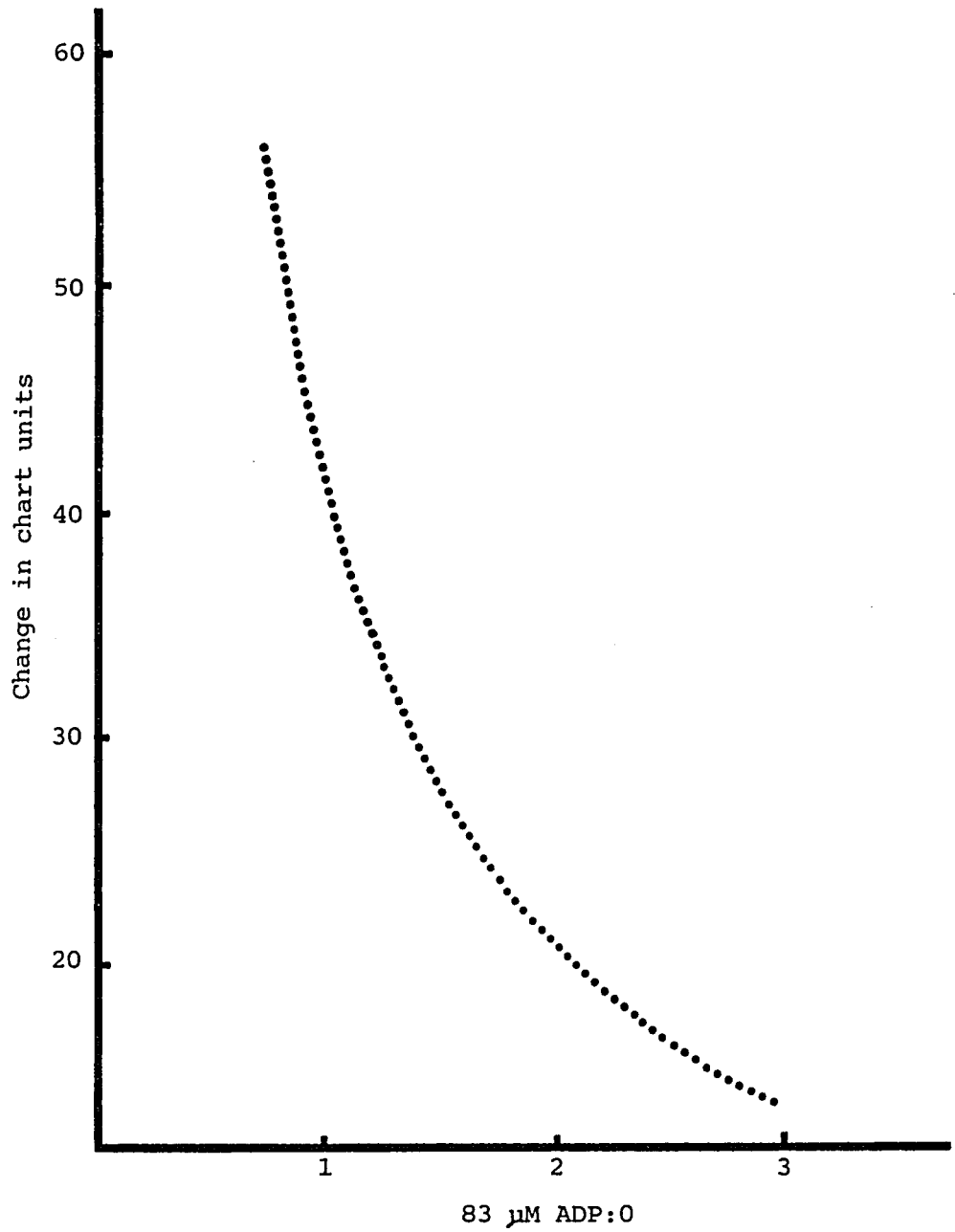

Figure 8. Relationship of oxygen uptake to ADP:0 ratio. Assumed oxygen concentration = 240 µM.

respiration rate, the error would be magnified with large differences in ADP:O ratio. However for this trait the true mean would be comparatively overestimated (i.e. enhanced ADP:O less likely to be detected). Figure 8 also points out the comparatively large effect on ADP:O ratio of chart measurement errors at high ratios of ADP:O. These examples underscore that predictions about characteristics of mixtures must be made carefully.

Together these results further substantiate the existence of the complementation phenomenon, and its linkage to combining ability of the source strains for a phenotypic trait of economic importance in livestock species. Sarkissian, Nicholas and McDaniel (1968) reported similar findings, as well as mouse mitochondrial respiratory heterosis, which was not found in hybrids in the present study. It can be concluded that complementation merits further study in economic animal species, especially with regard to potential application as a predictor of combining ability.

Unexpectedly, complementation was observed only in mixtures of mitochondria isolated from female mice. Sex-limited expression (or potential equivalent in plant species) of mitochondrial complementation has not been reported previously. Although the precise mechanism of mitochondrial complementation remains unknown, it can now be seen how gender dependence may have affected its

expression in previous animal experiments when gender differences were not tested.

Furthermore, across strains, mitochondria of female mice had significantly superior state 3 respiration rates, respiratory control ratios and ADP:O ratios, compared with those of male mice. Differences were most pronounced in the C57BL/6J inbred strain and with NAD-linked substrate. Androgen-mediated sexual dimorphism for ultrastructural and enzymatic features of mitochondria of mice and rats has been reported (Doeg et al. 1971; Doeg, Polomski and Doeg 1972; Koenig et al. 1980; Koenig, Goldstone and Lu 1980). However, based on these features, previous predictions had been that males would have superior respiratory capacity and ATP synthesis, the opposite of the present conclusions based on intact, respiring mitochondria.

To explore this discrepancy, androgen effects on mitochondrial respiratory capacity were directly evaluated. As expected from results of the present study, administration of testosterone to female mice tended to depress hepatic mitochondrial respiration rates (Table 11). State 4 respiration was depressed, relative to state 3, to the extent that respiratory control ratios tended to increase. The ADP:O ratio of females was unaffected by the brief testosterone treatment. Also as expected, orchiectomy tended to elevate hepatic mitochondrial respiration rate of male mice. Respiratory control ratios and ADP:O ratios

Table 11. Respiratory activities of hepatic mitochondrial fractions of testosterone-treated female, and orchiectomized male mice[a].

Group	State 3 ($\mu M\ O_2$/mg/min)	Respiratory Control Ratio[c]	ADP:O ($\mu M/\mu A$[c])	Final Body wt (g)[c]
Trial 1				
Control Males	4.63 ± .14	2.82[d] ± .04	1.46[d] ± .01	20.7[d] ± 0.4
Control Females	6.09 ± .03	3.44[d,e] ± .07	1.55[d,e] ± .02	17.3[d,e] ± 0.3
Testosterone-Treated Females	5.75 ± .52	3.61[e] ± .15	1.56[e] ± .00	18.5[f] ± 0.3
Trial 2				
Control Males	4.81 ± .32	2.78 ± .19	1.51 ± .08	25.3 ± 1.5
Orchietomized Males	5.06 ± .28	2.78 ± .04	1.51 ± .01	22.6 ± 0.8

[a]Data are means and standard errors of duplicate observations on groups of 3 individuals matched by initial weight, pooled over 3 respiratory cycles. C57BL/6J mice were littermates within trials. Exogenous substrate supplied was 2.5 mM pyruvate plus 2.5 mM malate. State 3 respiration was initiated repeatedly by addition of ADP to 125 µM.

[b]Initial to final ages were 37 to 44 and 57 to 64 days for Trials 1 and 2, respectively.

[c,d,e]Within trials, means with different superscripts are different ($P<.01$ for respiratory control ratio and ADP:O ration, $P<.05$ for final body weight) by t-test. Differences between control males and control females were not tested since degrees of freedom were distributed to tests of the treated group.

were unaffected by orchiectomy. Thus, despite predictions based on indirect evidence, these observations support the conclusion that isolated hepatic mitochondria of female mice show a definite respiratory advantage at the level of the intact organelle. Further studies over extended treatment periods and using mice of different ages may more completely elucidate hormonal regulation of mitochondrial bioenergetics. Such effects may be found to be of particular importance in livestock species, where orchiectomy is a common management practice during early stages of growth of market animals.

One novel candidate for an agent which could account for observed sex effects on mitochondrial respiratory capacity and complementation might be the intracellular fatty acid-binding proteins, recently reviewed by Bass (1985) and Glatz and Veerkamp (1985). These proteins, associated preferentially with mitochondrial membranes in vivo, bind free fatty acids to protect and regulate adenine nucleotide transport, acyl-CoA synthase, and the supply of fatty acids (Wu-Rideout, Elson and Shrago 1976; Barbour and Chan 1979) for a major process of mammalian mitochondrial energy transduction, beta-oxidation (Fournier and Rahim 1983, 1985). Significantly, these proteins are much more abundant in female rats than in males (Ockner et al. 1976, 1979, 1980; Alpers et al. 1983; Bass, Manning and Ockner 1983; Bass et al. 1985). Differences in fatty acid-binding

protein concentration among strains of rats and chicks have also been reported (Morrow, Allen and Martin 1979; Katongole and March 1980). Fatty acid-binding proteins have also been isolated from plants (Richers, Tober and Spener 1984). Observed mitochondrial respiratory capacity may thus reflect degree of protection during isolation and/or available endogenous substrate concentrations, both potentially mediated by fatty acid-binding proteins. Higher fatty acid-binding protein concentrations in females may also be found to favor mitochondrial interactions, including complementation. For example, mitochondria of a source comparatively richer in fatty acid-binding protein might be able to donate excess of the protein to a second source, by membrane contact in in vitro mixtures, enhancing activity of the recipient mitochondria. Also, Fournier and Rahim (1983, 1985) documented a capacity for self-aggregation of fatty acid-binding proteins, which might serve to hold mitochondria in close apposition, permitting contact-dependent interactions.

Hepatic Mitochondrial Activity and Performance of Cows

Dairy Cows

States 3 (10.74 $\pm$ 1.29 μM O_2/mg protein/min) and 4 (2.64 $\pm$ 0.49 μM O_2/mg protein/min) respiration rates of hepatic mitochondria isolated from lactating Holstein cows were lower than those of chickens and mice under similar

assay conditions. Respiratory control ratios (4.47 ± 0.41) and ADP:O ratios (1.22 ± 0.07 μM ADP/ μA O) were similar to those of chicken and mouse mitochondria utilizing succinate. The rate of ATP synthesis in these mitochondria (26.55 ± 3.48 μM ADP esterified/mg protein/min) was thus somewhat lower than that in chicken and mouse mitochondria. The necessary delay caused by transportation to the laboratory may have affected respiration rates, but all samples should have been affected similarly. Control experiments showed that the anesthetic lidocaine, a sodium channel blocker, had no effects on mitochondrial respiration in vitro.

No mitochondrial respiratory parameter was significant in an analysis of variance of milk production traits of cows and their parents. Linear (Table 12) and rank (Table 13) correlations of mitochondrial respiratory traits with milk production traits were calculated. Correlations with sire traits ranged about zero, as would be expected if cytoplasmic genetic effects were important. An interesting trend of positive correlations of dams' indices, and cows' indices and yields, with state 3 respiration rates, ADP:O ratios and ATP synthesis rates was observed. The agreement between correlations of dams' indices and cows' performance with mitochondrial activities was as would be expected if variation was due to maternally inherited cytoplasmic genetic effects. Correlations with mitochondrial

Table 12. Linear correlation coefficients of performance parameters with mitochondrial parameters of dairy cows[a].

Trait	State 3 Rate	Respiratory Control Ratio	ADP:O Ratio	ATP Synthesis Rate
Sire Predicted Difference Milk	.084	-.352	-.190	.033
Sire Predicted Difference Fat	.153	-.193	.093	.154
Dam Index Milk	.302	.399	.388	.303
Dam Index Fat	.105	.440	.026	-.057
Cow Index Milk	.304	.045	.246	.326
Cow Index Fat	.134	.089	.114	.124
Milk Yield[b]	.368	.053	.262	.479
Milk Fat Yield[b]	.083	.034	.082	.168

[a]Minimum significant ($P<.10$) $r = .549$ ($n=10$).

[b]Mean 305 d mature-equivalent yield

Table 13. Rank correlation coefficients of performance parameters with mitochondrial parameters of dairy cows[a].

Trait	State 3 Rate	Respiratory Control Ratio	ADP:O Ratio
Sire Predicted Difference Milk	-.230	-.412	-.267
Sire Predicted Difference Fat	.061	-.146	-.012
Dam Index Milk	-.212	.455	.406
Dam Index Fat	.121	.594	-.158
Cow Index Milk	.309	.285	.418
Cow Index Fat	.021	.324	.155
Milk Yield[b]	.430	.139	.394
Milk Fat Yield[b]	-.091	.030	.249

[a]Minimum significant ($P<.10$) $r = .550$ ($n=10$).

[b]Mean 305 d mature-equivalent yield

activities were consistently higher for indices and yields for milk than with those for milk fat. This result may be due in part to the relatively greater phenotypic variability for milk yield (coefficient of variation = 11.01) than for milk fat yield (308 $\pm$ 17 kg, coefficient of variation = 3.06) in this group of cows. These data tend to support the assertion (Bell, McDaniel and Robison 1985; Huizinga et al. 1986) that maternal breeding value may be more precisely estimated by partitioning cytoplasmic effects on performance variance. Comparative mitochondrial evaluation of individual animals should reflect the cytoplasmic genetic effects (a portion of maternal effects) and nuclear-cytoplasmic interaction effects specific to each animal (a portion of specific combining ability). These assays could be performed very early in life. Comparative values might then be included as a component of an index useful for predicting future performance of individuals and their progeny.

Beef Cows

Results of mitochondrial activity measurements on beef cows are presented in Table 14. No significant effect of breed was detected for any mitochondrial parameter estimated (Table 15). Linear models including breed, age, and day of lactation effects at best accounted for less than one third of the variation in any mitochondrial

Table 14. Respiratory activities of mitochondrial isolates from three breeds of beef cows[a].

Breed	$\mu M\ O_2$/mg prot/min State 3	$\mu M\ O_2$/mg prot/min State 4	Respiratory Control Ratio	ADP:O Ratio	ATP Synthesis (μM/mg prot/min)
Angus	17.2	4.1	4.2	1.18	40.6
Brangus	18.6	5.2	3.7	1.12	40.4
Hereford	17.3	4.2	4.3	1.21	41.4
Pooled SEM	0.6	0.2	0.7	0.01	3.8

[a]Data are means of 13 individuals per breed, pooled over two replications ($P>.10$) and three respiratory cycles.

Table 15. Results of repeated measures analyses of variance of mitochondrial parameters of beef cattle.

Model Effects	P>F State 3	State 4	Respiratory Control Ratio	ADP:O Ratio	ATP Synthesis Rate
Between					
Breed	.8299	.2065	.0939	.1022	.9608
Age	.9949	.2317	.0950	.3488	.8070
Day of Lactation	.7868	.2642	.0438	.0407	.4182
Within					
Trace	.4558	.2792	.7056	.5155	.5658
Cycle	.0001	.0001	.0001	.0211	.0001
Interactions					
Breed by Trace	.3659	.3494	.1994	.0458	.2345
Breed by Cycle	.0911	.0286	.8059	.9320	.8074

parameter. It is possible that heterogeneous mitochondrial populations exist among breeds, observed as maternal genetic effects, but exerting their influence in ways other than via respiratory metabolism. Brangus cows tended to have higher state 3 respiration rates, but lower ADP:O ratios, resulting in uniform ATP synthesis rates. These ATP synthesis rates were greater than those observed for dairy cattle, and intermediate to those of chickens and mice under similar assay conditions. As wet tissue weights could not be obtained, mitochondrial yields were not estimated. The covariate, day of lactation, appeared to have an effect on mitochondrial respiratory control ratios and ADP:O ratios ($r = +.20$ across breeds for each parameter), though differences were not due solely to a difference between dry and lactating cows. In contrast to chicken mitochondria, which tended to have increasing activity rates in successive respiratory cycles, and to mouse mitochondria which had no consistent trends across respiratory cycles, beef cow mitochondria exhibited activities which consistently decreased with successive respiratory cycles.

Stepwise regression of weaning and yearling growth traits of cows, their collateral relatives and progeny on mitochondrial traits revealed mainly nonsignificant, small mitochondrial effects. Significant negative standardized regression coefficients were observed for some weaning

growth traits of Angus and Brangus cows regressed on ADP:O ratios (Table 16). Unexpectedly these suggested cows with heavier weaning weights tended to have less efficient hepatic mitochondria. No other pattern of mitochondrial effects was detected. These findings, which must be regarded as preliminary since sample size was so small, suggest variability in hepatic mitochondrial respiratory parameters has only little association with weaning and yearling growth traits of beef cattle. This is also in contrast to the pattern of relationships, though statistically non-significant, between lactation performance (an important determinant of weaning weight) and hepatic mitochondrial respiratory capacity observed in dairy cattle. Inbred and hybrid mice with comparatively heavy body weights did not have superior mitochondrial respiratory activity, however. The limited range of phenotypic variation in beef cattle available for sampling (growth breeding value range 95 - 107 for Angus and Brangus, and only 98 - 106 for Hereford) very likely restricted abilities to test correlations with mitochondrial function. Non-mitochondrial, cytoplasmically transmissible agents (infectious agents) may deserve more scrutiny if important maternal cytoplasmic effects on performance are widely substantiated.

The composition (relative donor proportions) of complementation mixtures of Angus and Hereford mitochondria

Table 16. Significant standardized regression coefficients from stepwise regression of performance parameters on mitochondrial parameters of beef cows[a].

Dependent Variable	State 3 Rate	RC Ratio	ADP:O Ratio
Cow weaning weight ratio	-.64* (A,1)		-.43* (A,1)
Partial r^2	.347		.252
Progeny weaning weight ratio		-.83* (B,1)	-1.05* (B,3)
Partial r^2		.689	.672
Cow growth breeding value		-.85* (B,1)	-.94** (B,2)
Partial r^2		.725	.951
Cow yearling weight ratio	-.49+ (A,1)		
Progeny yearling weight ratio	-.40* (B,3)	-.83** (B,1)	-.94** (B,2)
Partial r^2	.435	.650	.142
Maternal half-sib yearling weight ratio		-.82* (H,1)	
Partial r^2		.446	

[a]Data are the greatest significant (+ = P<.10, * = P<.05, ** = P<.01, *** = P<.001) coefficients observed for each (breed, cycle) cell. A = Angus, B = Brangus, H = Hereford. Three cycles were recorded in duplicate on each animal. No significant correlations were found with State 4 respiration, or for cow maternal weaning or yearling weight breeding value, maternal half-sib weaning or yearling weight ratio, or paternal half-sib traits of each breed with any mitochondrial trait.

was determined retrospectively from protein analyses. Since the proportions were not precisely 1:1 (though no mixture exceeded 1.5:1), significance of complementation could not be tested by linear contrasts. Chi-square analyses detected no mitochondrial interaction for state 4 respiration rates, respiratory control ratios or ADP:O ratios (X^2 = 8.950, 0.459 and 0.089, respectively). Mean state 3 respiration rates of mixtures were significantly lower than predicted (X^2 = 62.783, $P<.01$), probably due to non-homogeneous source suspensions (for example, the correlation between protein concentrations of first and second reaction mixture replicates = +.82), though this result could also be construed to reflect negative complementation. Negative complementation would be inconsistent with complementation theory in this instance, in view of reported enhanced respiration of cells of hybrid cattle (Skulmowski 1975) and heterosis of Angus X Hereford cattle (Long 1980). A more thorough test of complementation of mitochondria of cattle might depend on use of breeds exhibiting more between-breed variability in mitochondrial activities.

SUMMARY AND CONCLUSIONS

A pilot study using broiler and layer chickens demonstrated the feasibility of surveying interstrain variation in mitochondrial respiratory capacity of animals. Differences in mitochondrial activity parameters between chicken strains were small but consistent across respiratory cycles. No associations of mitochondrial respiratory activity with within-breed variation in body weight or egg production rate was detected.

Previously unsurveyed, significant differences among strains of genetically standardized inbred mice, in state 3 respiration rate, respiratory control ratio and ADP:O ratio, were observed. Rapid ATP synthesis rate appeared to be positively associated, in a general way, with superior reproductive capabilities, but not with large body size. An unexpected sexual dimorphism in mitochondrial respiratory capacity was observed in these mice. Direct assessment of androgen effects supported the conclusion, contrary to previous predictions, that females exhibited an advantage in respiratory capacity at the level of the intact organelle.

Hybridization resulted in apparently increased mitochondrial mass (increased amount of protein in the mitochondrial fraction of cell homogenates), but not in

enhanced mitochondrial respiratory activity of two genotypes of hybrid mice. Original breeding data confirmed one hybrid used in mitochondria studies tended to exceed midparent for preweaning growth, and was significantly heterotic for postweaning growth, while the other hybrid used was not heterotic for any trait. Thus there was no apparent association between expression of heterosis for growth traits, which are of economic importance in livestock species, and mitochondrial function in mouse hybrids used as models for such species.

A striking pattern of positive correlations (though none reached statistical significance with the present sample size) of ATP synthesis rates of Holstein dairy cows with milk production indices and yields of cows and their dams was observed. This suggested maternal breeding value for milk yield might be more precisely predicted by partitioning cytoplasmic effects on performance variance. No breed differences in mitochondrial respiratory parameter estimates were detected among Angus, Brangus and Hereford beef cows. Also, no consistent pattern of associations with weaning or yearling growth traits of beef cows was observed. These data constitute the first comparative study of mitochondrial respiration and oxidative phosphorylation of breeds of cattle ever reported.

Consistent with complementation theory, no mitochondrial complementation was observed for chicken

strains with neutral combining ability. Significant complementation was detected for state 3 respiration rate and respiratory control ratio of mitochondria of females from mouse strains with positive combining ability for growth. No complementation was observed for males or any combination of neutrally combining strains. These results were also consistent with complementation theory, and further substantiated existence of the complementation phenomenon. Complementation by mitochondria of females exclusively was unexpected, and this finding may help guide future exploration of mechanisms of complementation. State 3 respiration rates only of mixed cattle mitochondria were lower than expected, but this was attributed to uncertainty in mixing rather than negative complementation.

Though experiments were not conducted so as to permit rigorous between-species comparisons, mice ranked highest for mitochondrial ATP synthesis rates, followed by beef cattle. Dairy cattle ranked lowest for mean mitochondrial ATP synthesis rates.

Further comparative studies on variation in mitochondrial respiratory metabolism seem justified, especially with regard to lactation of cattle. Also these results should help justify further attempts to partition cytoplasmic effects on performance variance, as well as investigations of the effects of mitochondrial DNA polymorphisms on mitochondrial and cellular function.

APPENDIX A

STEPWISE SEPARATION OF MITOCHONDRIAL FRACTIONS FROM CELL LYSATES

1. Prepare suspension of cell lysate or tissue homogenate in 30 ml grinding buffer.

2. Filter through nylon gauze to remove hairs, blood clots, connective tissue, etc. Decant into 50 ml polycarbonate centrifuge tubes. Centrifuge at 600 g (2250 rpm in Sorvall SS-34 fixed-angle rotor) for 5 min (deceleration brake off).

3. Decant supernatant into clean polycarbonate tubes and centrifuge at 10,000 g (9250 rpm) for 10 min.

4. Discard supernatant. Wipe any lipid adhering to tube walls. Gently resuspend pellet in 5 ml grinding buffer. Centrifuge at 12,000 g (10,000 rpm) for 10 min.

5. Repeat step 4.

6. Discard supernatant. Resuspend pellet to yield approximately 1 mg mitochondrial protein/ml.

   Note: All operations are conducted at 0-4 C using prechilled reagents and equipment. Erythrocyte contamination can be minimized by centrifugation at 8,500 g (8000 rpm) for 5 sec and transfer of supernatant to a clean tube before continuing step 3. Composition of buffers is given in Table 2.

APPENDIX B

METHOD FOR TOTAL PROTEIN QUANTITATION

Stock Color Reagent

100 mg brilliant blue G
50 ml 100% ethanol
100 ml phosphoric acid
H_2O q.s. to 200 ml

Working Color Reagent

1 part stock + 4 parts H_2O, filtered through #1 filter paper

Stock Standard

2.000 mg/ml fatty acid-free BSA

Standards

µg/cuvette	µl std/cuvette	µl H_2O/cuvette
0	0	100
20	10	90
50	25	75
100	50	50
150	75	25

Procedure

Add 5 ml working color reagent/cuvette. Mix well and stand 5 min. Determine absorbance at 595 nm.

Reference: Bradford 1976.

Samples

Use 10 µl samples of mitochondrial suspension, disrupted by freezing, or preferably mechanically homogenized.

APPENDIX C

PRECISE PROTEIN QUANTITATION FOR COMPARISON OF MITOCHONDRIAL RESPIRATION

Mitochondrial steady state respiration rates must be expressed on a specific activity basis for comparative purposes. Typical units are O_2 uptake/mg mitochondrial protein/min. However, mitochondria are commonly isolated by differential centrifugation using albumin-containing buffers. The possibility was considered that in consecutive washings the mitochondria may become "coated" with albumin adsorbed to fatty acid moieties on membrane surfaces. If albumin was carried over from step to step in mitochondrial pellets during isolation, mitochondrial protein (for example, yield per unit wet tissue) would be overestimated, and specific activities underestimated by simply subtracting the amount of albumin supplied by the final resuspending buffer from the final total protein concentration.

To explore this possibility, respiration rates of 92 samples of bovine hepatic mitochondrial fractions isolated by differential centrifugation in 0.5 percent w/v bovine serum albumin (BSA)-containing buffer were compared. As the standard method, steady state respiration rates were calculated from O_2 uptake/min slopes based on mitochondrial

protein estimated as described above. Total protein in each suspension was estimated by the method of Bradford (1976). Each sample was then subjected to polyacrylamide gel electrophoresis. Gels were stained, and the intensity of each protein band was measured at 633 nm using an LKB Ultroscan scanning laser densitometer. The resulting absorbance profile of each sample was integrated and normalized to a cumulative relative area of 100 percent. Protein bands in each sample corresponding to albumin were identified by comparison to a BSA standard (Figure A.1) and subtracted. The residual area percent constituted the fraction of the total which was non-albumin, or mitochondrial protein. Steady state respiration rates were recalculated from the same O_2 uptake/min slopes using these precise mitochondrial protein estimates.

The standard method comparatively overestimated the amount of mitochondrial protein present in the suspensions by a mean 18.0 $\pm$ 3.5 percent. Therefore mean respiration rates were comparatively underestimated by standard calculations. Some samples were overestimated by as much as 160 percent, suggesting significant albumin carryover. However, some samples proved to be comparatively underestimated by the standard method, by up to 52 percent. This was probably due to the volume occupied by the mitochondrial pellet itself, which had been assumed to be negligible, constituting an important proportion of the

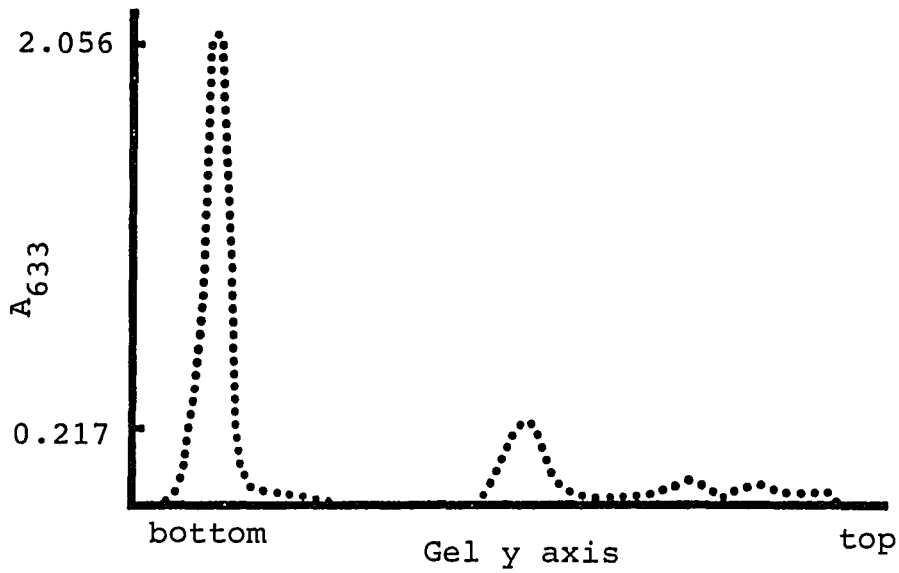

Figure A.1. Integration profile of albumin protein standard.

final volume in comparison to the volume of the resuspending buffer. This conclusion was supported by the negative linear correlation ($r = -.34$, $P<.01$) observed between amounts of BSA and mitochondrial proteins across all samples. If carryover from step to step during isolation by albumin adsorption to mitochondria was occurring, this correlation would be expected to be positive. Therefore buffer nonspecifically adsorbed to the final mitochondrial pellet before resuspension, not removable by decanting, may contribute misleading amounts of BSA to the final protein total. These results document the error that may arise from calculating specific respiration activities using the standard method.

Table A.1. Protein Electrophoresis[a].

Solution	Components
A	18.1 g tris base + 0.4 ml Triton X-100 / 100 ml, pH 8.8
B	30.0 g acrylamide + 0.8 g bisacrylamide / 100 ml
C	0.2 g ammonium persulfate / 10 ml, freshly made
D	6.2 g tris base + 0.16 ml Triton X-100 / 100 ml, pH 6.8
E	4.5 g tris base + 21.25 g glycine + 1 ml Triton X-100 / 1, pH 8.3
F	tetramethylenediamine (TEMED), 6.6 M

gel	ml H_2O	ml A	ml B	ml C	ml D	ml E	ml F
comb	15.6	0	5.0	1.7	0	7.5	0.02
stacking	15.6	0	5.0	1.7	7.5	0	0.02
running	16.1	10.0	13.4	0.6	0	0	0.01

[a]Gels were run for 1 hr with constant current at 1 watt/1.5 mm gel, followed by 4 hr at 3 watts/gel. Temperature was controlled at 5 C. Solution E was upper and lower tank buffer. For staining, gels were prefixed in 12% w/v trichloroacetic acid (TCA), then stained overnight in fresh 0.1% w/v brilliant blue G + 2% w/v TCA + 2% w/v ammonium sulfate. Gels were destained by washing 3 min in 0.1 M tris base buffer (pH adjusted to 6.5 using phosphoric acid), followed by a 1 min rinse in 25% v/v methanol. Gels were preserved in 20% w/v ammonium sulfate (Neuhoff, Stamm and Eibl 1985). In preliminary practice, background staining was unsatisfactory in gels stained with 0.25% w/v brilliant blue R or G in 50% v/v methanol-10% v/v acetic acid, destained with 7.5% v/v acetic acid-5%v/v methanol. Also, silver staining subsequent to staining as described above did not enhance band visibility.

APPENDIX D

MOUSE HUSBANDRY

Inbred A/J, C57BL/6J, BALB/cJ and DBA/2J mice were obtained from The Jackson Laboratory. Mice were housed in 5700 cm3 plastic cages with woven wire lids and contact bedding. Cages were cleaned weekly with disinfectant. Approximate room temperature was maintained. Subdued natural lighting was present. Incoming room air was filtered. Vapor pesticide strips were provided. A complete pelleted ration (Rodent Blox, Wayne Feeds) and tap water were provided ad libitum.

One male and one female, chosen randomly from the required strain, were together continuously once paired, to afford opportunity for maximum reproductive and lactational stress on the females. Parents were not precisely matched by initial age but were under 4 mo old. Cages were checked daily on weekdays for new litters. Mice were group-weighed using a triple-beam balance accurate to 0.1 g. Mice were weaned at 3 wks, and caged together as littermates regardless of litter size until reaching 5 wks of age. Mice reserved for future matings were caged together by sex and genotype. Discarded mice were euthanized by exposure to carbon dioxide.

The only phenotypic abnormality noted during the study was idiopathic alopecia, of two types. Caudal alopecia was observed in both pre-weaning and mature mice of both sexes of several genotypes, possibly due to auto- or allogenic grooming behavior. This had no apparent effect on growth. Facial alopecia, including loss of vibrissae, was observed in a few female mice of different genotypes, nursing large litters. This could have been due to grooming behavior or stress. During the study several mice were found with missing limbs or tails, probably due to injury.

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