REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 1998	Technical Report

4. TITLE AND SUBTITLE
Thermoregulatory, endurance and ultrastructural effects on acute and subchronic pyridostigmine bromide administration in the exercising rat

5. FUNDING NUMBERS

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Same as 7 Above

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Pyridostigmine (PY) is the drug currently approved for chemical agent prophylaxis. Previous studies have demonstrated that acute administration or high doses of PY have resulted in thermoregulatory, endurance and ultrastructural abnormalities. In this study the effects of acute (APY, 100 ug/kg, iv) and subchronic PY for 2, 3, and 4 weeks (PY2, PY3, PY4, 20 ug/hr, via osmotic pump) administration on treadmill endurance when measured in rats to determine whether any voluntary muscle weakness occurs as a result of PY administration. APY rats had reduced endurance time (56 $\pm$ 18 min (mean $\pm$ SD) vs 72 $\pm$ 17) compared to saline controls and increased core temperatures at the start of exercise (Tc, 38.9 $\pm$ 0.2oC vs 38.5 $\pm$ 0.6oC). These decrements were ameliorated with subchronic administration, and PY2 animals maintained lower Tc than SAL2 animals. Typical mitochondrial lesions detected with acute high doses of PY (1mg/kg were not observed in any of these specimens. Decrements in endurance and thermoregulation and ultrastructural abnormalities previously seen with acute PY are not evident after subchronic administration for up to 4 weeks.

14. SUBJECT TERMS
Pyridostigmine, exercise, rat, anticholinesterase, ultrastructure, chronic, performance, acute

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

TECHNICAL REPORT
NO. T98-11

Thermoregulatory, endurance and ultrastructural effects of acute and subchronic pyridostigmine bromide administration in the exercising rat

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Project Reference: 3M162787A875

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EXECUTIVE SUMMARY

Pyridostigmine (PY) is the drug currently approved for chemical agent prophylaxis. Previous studies have demonstrated that acute administration or high doses of PY have resulted in thermoregulatory, endurance and ultrastructural abnormalities. In this study the effects of acute (APY, 100 ug/kg, iv) and subchronic PY for 2, 3, and 4 weeks (PY2, PY3, PY4, 20 ug/hr, via osmotic pump) administration on treadmill endurance was measured in rats to determine whether any voluntary muscle weakness occurs as a result of PY administration. APY rats had reduced endurance time (56 ± 18 min (mean ± SD) vs 72 ± 17) compared to saline controls and increased core temperatures at the start of exercise (Tc, 38.9 ± 0.2°C vs 38.5 ± 0.6°C). These decrements were ameliorated with subchronic administration, and PY2 animals maintained lower Tc than SAL2 animals. Typical mitochondrial lesions detected with acute high doses of PY (1mg/kg) were not observed in any of these specimens. Decrements in endurance and thermoregulation and ultrastructural abnormalities previously seen with acute PY were not evident after subchronic administration for up to 4 weeks.

INTRODUCTION

Anticholinesterase poisoning leads to the accumulation of the neurotransmitter acetylcholine (ACh) at cholinergic effector sites due to the inhibition of the enzyme responsible for its destruction, acetylcholinesterase (AChE). Severe poisoning can result in death from respiratory arrest due to uninterrupted firing of central and peripheral nerves controlling respiration (4). Pretreatment of animals with reversible inhibitors of cholinesterase such as the carbamates pyridostigmine (PY) or physostigmine (PH) reduces the lethality due to exposure to irreversible organophosphorus (OP) anticholinesterases (antiChE) such as the chemical warfare nerve agents soman, sarin, tabun and VX.

Pyridostigmine bromide (PYBr) has been chosen as the U.S. military's standard pretreatment for OP anticholinesterase chemical warfare agents. Pyridostigmine is used clinically in the treatment of myasthenia gravis, because its nicotinic stimulatory effects at the neuromuscular junction (NMJ) and direct contractile effects are of benefit to myasthenics (11), but may result in performance decrements with acute administration (9,29). Muscarinic side effects of pyridostigmine include increased intestinal motility, salivation, lacrimation, bronchial secretions, and meiosis (11). Alteration of cholinergically-mediated thermoregulatory processes has been demonstrated. Hyperthermia is a commonly reported symptom in cases of anticholinesterase poisoning, and the hyperthermia is positively correlated with the % ChE inhibition (32). The hyperthermia is consistent with thermoregulatory decrements elicited by acute PYBr administration in the heat (9,21,29). Since as a nerve agent prophylaxis PY may be administered to healthy individuals, a great deal of recent interest has focused on the behavioral, pathological, and physiological consequences.

There is much evidence that acute, subchronic (administration for a time span of a few days to 10% of the life span of the animal), and chronic exposures to cholinergic compounds result in differing effects (6,11,31). Acute, but not subchronic, administration of PH resulted in decreased endurance in a running rat model (25,26,28). Several, but not all, of soman's behavioral, enzymatic, and physiological effects were reversed upon repeated sublethal dosing (31). Since the doctrinal usage of PYBr as a pretreatment involves sustained dosages (30 mg tablets t.i.d. for 2 weeks), the potential for adverse side effects in healthy individuals should be examined in both subchronic and acute dosing regimens.

Thermoregulatory decrements induced by acute administration of PYBr were attenuated with subchronic administration in sedentary patas monkeys (3,7). Acute administration of PYBr resulted in an increased rate of rise of core temperature (Tc) in rats exercising at an ambient temperatures (Tamb) of 35°C with 60% ChE inhibition (9) and at 26°C with 40% ChE inhibition (29). However, subchronic (oral, in drinking water) administration of PYBr at doses that elicited a ChE inhibition of up to 40% resulted in no decrements in rats exercising in the heat (35°C) (10). Subsequent studies with acute (26,28) and subchronic (25) PH (carbamate with central as well as peripheral sites of action) administration also indicated that decrements resulting from acute administration were attenuated with subchronic administration.

Kolka and Stephenson (21), Seidman and Epstein (32), and Epstein *et al.* (8) measured

thermoregulation in exercising, heat-exposed human subjects following PYBr administration. A single 30 mg tablet of PYBr followed by 15 min of rest and 30 min of bicycle exercise at 22, 29 and 36°C, 30% rh, resulted in decreased skin blood flow and heart rate and increased esophageal temperature at 29 and 36°C (21). Kolka and Stephenson (21) concluded that the reduction in skin blood flow resulted in increased heat storage which could decrease thermal tolerance in hot environments or when wearing chemical protective clothing. However, Seidman and Epstein (32) observed no differences in cardiovascular variables or heat storage during 2 hr of treadmill walking (30°C, 60% rh) between control subjects and those receiving 4-30 mg doses of PYBr 8 hr apart. Later, Epstein *et al.* (8) observed no differences between control and PYBr-treated (4-30 mg tablets) subjects exercising at 30°C, 60% rh in chemical protective clothing.

No decrements in thermoregulation or endurance of humans (8,32), monkeys (3) or rats (10) were reported with subchronic PYBr administration (40% ChE inhibition) and elevated Tamb (30°C or higher). However, recent work from this laboratory (23) examined the effects of Tamb and PH administration on performance and thermoregulation. Decrements with acute PH were evident at Tamb of 15 and 26°C but not at 10 or 30°C. We concluded that 30°C may be too high a Tamb for effective thermoregulation in either saline-treated controls or PH-treated exercising rats, and analogously, 10°C may be cool enough to allow sufficient radiant heat loss to compensate for the increased metabolic heat generation in PH-treated rats. Therefore, the lack of thermoregulatory decrements with subchronic PYBr administration may have had as much to do with the Tamb as any accommodation to the drug. The effects of acute and subchronic administration of PYBr on thermoregulation in the running rat model need to be re-examined using a temperature range in which differential effects were seen with PH.

Acute PYBr was found to elicit ultrastructural alterations at the NMJ of rat diaphragm muscle (5,14), but these alterations were reversed with subchronic administration (5). Additionally, work from our laboratory (25) with PH indicated that ultrastructural changes and performance decrements seen with acute administration were not observed after 2 week subchronic administration. However, Adler *et al.*(1), reported that while there was no alteration in diaphragm function, there was alteration of function in skeletal muscle stimulated *in situ* but not *in vitro* throughout 2 weeks of PY administration. Therefore, the current study was designed to examine skeletal muscle function and ultrastructure following subchronic (4 week) PY administration at ChE inhibition levels of 30-40% as achieved by administration of 30mg three times a day in man (15,19,20,33).

METHODS

Animals Adult male Sprague-Dawley rats (Charles River, CD strain, 510-530g) were caged individually in wire-bottomed cages in an environmental chamber (4 x 3 x 2 m) at 26°C and 50% rh, and used one time only. Lighting was controlled automatically (on, 0600-1800 h) and Purina rat chow and water were available *ad lib* except during experimental intervals.

Drugs The form of PY used was pyridostigmine bromide (ICN Biochemicals, Cleveland, OH, Lot# 32274, obtained from and tested for purity by Walter Reed Army Institute of Research). For the acute experiments, 100 ug/kg PYBr was dissolved in 0.2 ml of sterile 0.9% saline and

administered via lateral tail vein (iv). In the 2 week subchronic experiments, 20 ug/hr PYBr was administered (5 ul/hr) via osmotic mini-pump (model 2ML2, Alza, Palo Alto, CA), or for the 3 and 4 week studies the same 20 ug/hr was administered (2.5 ul/hr) via osmotic mini-pump (model 2ML4). Osmotic pumps were implanted subcutaneously under methoxyflurane anesthesia according to the procedure outlined by the manufacturer.

Cholinesterase (ChE) inhibition To approximate acute and subchronic doses of PY to elicit a 40% inhibition of whole blood ChE, a range of doses was tested using 3 rats per dose. Once appropriate doses were established, numbers of animals in each of 5 groups (saline iv, PY iv, saline via osmotic pump, PY via osmotic pump 2 and 4 week) were increased.

To determine % ChE inhibition in the acute studies, a blood sample (0.3 ml) was drawn prior to PY injection and again one hour post injection. In the subchronic experiments, a blood sample was drawn from the tail vein prior to osmotic pump implantation and additional 0.3 ml samples were drawn 1, 7, 14, 21, and 28 days after implantation. All samples were analyzed for whole blood ChE activity using our modification (24) of the Boehringer Mannheim Diagnostics' ReagentSet Cholinesterase #124117.

Experimental design Following dose determination, 7 groups (N=12 per group) of rats were treated as follows:

A) 0.2 ml saline via tail vein (ASAL)
B) 100 ug/kg of PYBr in 0.2 ml of saline via tail vein (APY)
C) saline via osmotic pump for 2 and 4 weeks (SAL2 and SAL4)
D) subchronic PYBr (20 ug/hr) via osmotic pump for 2, 3, and 4 weeks (PY2, PY3, PY4).

Fifteen min following acute drug administration or 2, 3 or 4 weeks following osmotic pump implantation, the rats were run on a treadmill. At the end of exercise, the animals were anesthetized and muscles removed for ultrastructural examination.

Exercise procedure All rats (naive, untrained) were run (11 m/min, 26°C, 50% rh, 6° incline) on a motor-driven treadmill with a shock avoidance contingency. They were allowed a 2 min rest at 20 and 40 min. Then, they were run to exhaustion, which is the point at which the rat is unable to keep up the pace and/or, when placed on its back, will not right itself. A trained technician was present at all times to observe the animals closely and remove animals from the treadmill as necessary. This same protocol has been used and validated for consistency in previous studies (12,13,22,28,29). During the run and subsequent recovery period, core (Tc, rectal probe, 6.5 cm beyond anal sphincter) and tail (Tt, mid-dorsal) skin temperatures were monitored continuously with an automated data acquisition system.

Ultrastructural evaluation Three different muscles were examined: the diaphragm, a mixed fiber type muscle; the soleus, a red or slow twitch fiber type muscle; and the extensor digitorum longus, a white or fast twitch muscle type. For these assessments 6 of the 12 rats in each of the APY and PY4 groups and an additional 6 rats that were neither treated with PY nor run (controls for ultrastructural evaluation) were used. The animals were anesthetized with methoxyflurane 30 min after the end of exercise. The diaphragm, soleus, and extensor digitorum longus muscles

were removed, fixed, processed for transmission electron microscopy (EM),and evaluated for focal or generalized changes.

Diaphragm muscle of anesthetized rats was bathed *in situ* with a total of 5ml of Karnovsky's fixative (18) delivered through a 21 gauge needle on a 10cc syringe, rapidly excised, fixed for an additional 2 hr, and 1mm strips stained to reveal neuromuscular junctions (NMJ) (17). The same procedure was applied to soleus and extensor digitorum longus muscles except that prior to excision they were clamped to a rigid support to prevent changes in length during fixation. Specimens were post-fixed for 2 hr with 1% osmium tetroxide in 0.052 M cacodylate-6% sucrose buffer (4°C) and processed for electron microscopy. Tissues from each rat were examined for the presence of multiple spherical areas of low electron density in mitochondria at NMJ. Positive and negative controls for PY-induced mitochondrial lesions were obtained with diaphragm muscles from rats receiving subcutaneous mid-back injections of 1mg/kg PY (Mestinon) or equivalent carrier buffer.

Statistics- Statistical comparisons between each PY group and its corresponding SAL group (Tables 2-4) were done using Student's non-paired "t" test. The Tc and Tt data (Fig. 1, Fig. 2) were analyzed by group and by time using a repeated measures analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons. The null hypothesis was rejected at $p < 0.05$.

RESULTS

Table 1. % ChE Inhibition

Group	1 hour (Acute)	1 day	1 week	2 weeks	3 weeks	4 weeks
SAL	6 ± 17 (8)	5 ± 15 (6)	-3 ± 21 (6)	3 ± 20 (6)	---	---
PY	44[a]± 5 (10)	51[a]± 12 (20)	34[a]± 30 (20)	43[a]± 15 (20)	37[a]± 13 (10)	50[a]± 13 (10)

Values are mean ± S.D. (N)
a Significantly ($p<.05$) different from saline controls.

Whole blood ChE activity was determined prior to administration of SAL or PY and at the indicated time (Table 1) following administration. Table 1 contains the % ChE inhibition data for rats given ASAL or APY (100 ug/kg) or SAL or PY (20ug/hr) via osmotic pump for 1, 2, 3, or 4 weeks. There was a significant inhibition (range 34 to 51%) in all groups receiving PY.

A summary of the exercise data for all groups (Table 2) indicates that the only groups with significantly ($p<.05$) decreased endurance (run time) was the APY group compared to

ASAL; the APY group also had an elevated Tc at the start of run (SOR). After 2 weeks the only difference between the SAL2 and PY2 groups is that the PY2 group had a significantly lower TcEOR (end of run) ($p<.02$) despite very similar endurance times. The Tc SOR of the SAL4 group was significantly lower than that of the PY4 group.

Tables 3 and 4 contain the mean Tc and Tt data for all groups at intervals from the SOR until 4 of the animals in each group had reached exhaustion. Using Student's "t" test to compare values in PY and corresponding SAL groups, the only additional differences noted were the higher Tc's of PY4 animals when compared to those of SAL4 animals (through 20 min). Further, the Tt of PY2 animals was lower than that of the SAL2 animals at 28 and 36 min of run.

In Fig. 1 the Tc of ASAL vs APY (Fig 1A) and SAL2 vs PY2 (Fig 1B) are graphed from the SOR until the time that 4 of the 12 animals in each group had reached exhaustion. See Table 3 for the number of animals at each point. Figure 2A and B contain the corresponding Tt data. A repeated measures ANOVA by group indicated that there was a significant ($p<.01$) difference between groups for Tt at both acute and 2 week PY administration and for Tc at 2 weeks but not acutely. After 2 weeks of PY administration the animals were able to maintain a significantly lower Tc throughout the run than animals given SAL. APY animals had higher Tt than ASAL, and PY2 were lower than SAL2 throughout the run. There were no consistent differences in Tc or Tt after 4 weeks of administration (see Tables 3 and 4).

Rats do not sweat, but they do evaporatively cool by spreading saliva on their ventral surfaces. While running on a treadmill, they are unable to spread saliva, but it is still secreted and dehydrates the rat. The weight loss per minute of run was significantly greater in APY compared to ASAL (0.32 ± 0.06 vs 0.24 ± 0.04 g/min), but there were no significant differences at 2 or 4 week between SAL and PY rats (data not shown).

Fig. 3 illustrates the typical multiple spherical areas of low electron density in mitochondria lesions produced at NMJs in diaphragm muscle cells by high acute doses of PY (positive control). Fig. 4 illustrates typical mitochondrial at NMJs in normal diaphragm muscle (negative control). Fig. 5 shows normal mitochondria in soleus muscle of rat after acute exposure to PY (APY, 100μg/kg) and run to exhaustion as indicated under the exercise procedure. Fig. 6 shows normal mitochondria in soleus muscle of rat after a four week exposure (PY4). Note in Fig. 5 and 6 that the combination of exercise and acute or subchronic PY administration elicited no abnormalities in mitochondrial ultrastructure. Stain deposits used to localize NMJs are also evident in this micrograph. Fig. 7 shows normal mitochondria in diaphragm (PY4). None of the other specimens observed showed PY-induced mitochondrial changes.

DISCUSSION

The decreased endurance and increased Tc with APY observed in this work are consistent with earlier reports (3,7,9,29). Moreover, Winger et al. (34) reported that multiple doses of PY (t.i.d. for 7 days) resulted in slight improvements in thermoregulation during a heat stress test as compared to slight decrements after a single dose (21). In sedentary heat-stressed rats, a 2 week PY regimen (same dosage and route as in the current work) induced a state similar to heat

acclimation: PY2 rats maintained lower Tc and increased evaporative water loss resulting in increased heat endurance (27). In the present work the PY2 group also had a consistently lower Tc than the SAL2 group.

Neither acute nor subchronic (four week) exposure to PY at the doses utilized in these experiments produced typical mitochondrial lesions detected in the positive control. Earlier work demonstrating myopathy or neuropathy ordinarily employed higher levels of ChE inhibition and also showed distinct fasciculations which by themselves have been shown to induce these anomalies (2).

Most adverse effects of PY are associated with doses resulting in greater that 40% ChE inhibition (5,9,14) or acute (3,7,9,21,29) administration or both. With subchronic administration at $\leq$ 40% ChE inhibition thermoregulatory decrements, especially in the heat, have been eliminated (3,5,8,10,32). The present study extends these observations on the attenuation of decrements with chronicity in the case of treadmill endurance tests at 26°C.

In the time since the end of the Persian Gulf War many veterans of that conflict have presented with diverse and unexplained symptoms such as fatigue, joint pain, skin rash, shortness of breath, etc. While no single cause has as yet been able to explain these symptoms, subchronic administration of PY at approved dosages was hypothesized as one of the possible etiologies (16,30). However, in this study we were not able to demonstrate any effects on the ultrastructure or function of the neuromuscular junction when similar levels of ChE inhibition were achieved.

ACKNOWLEDGMENTS

The authors would like to thank SGT's Anthony I. Osagie and Daniel K. Navara and Mr. Michael Blaha, Ms Kimberly A. Tartarini and Dr. John Kohl for their technical support.

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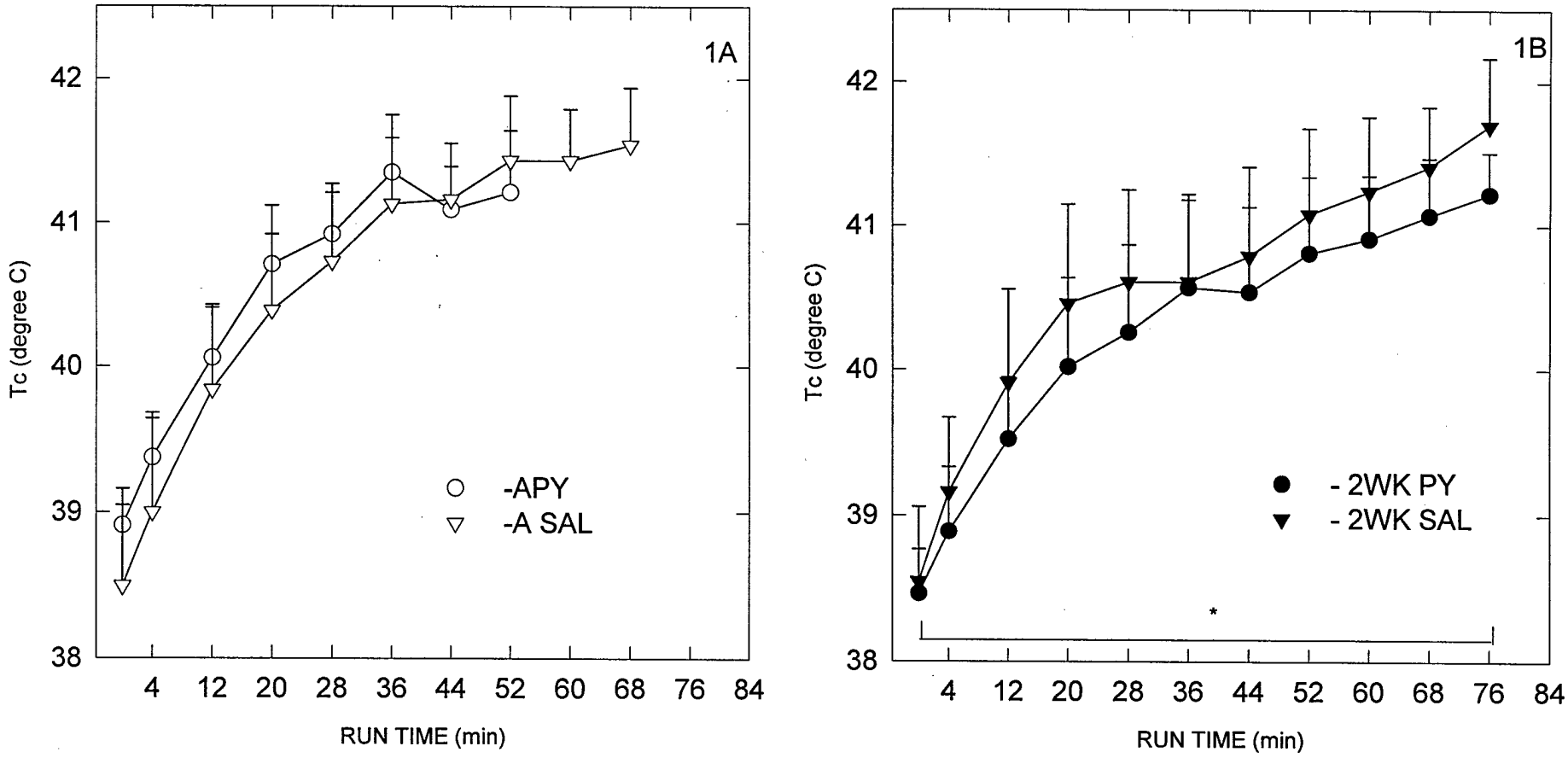

Fig. 1- Tc of ASAL vs APY (Fig 1A) and SAL2 vs PY2 (Fig 1B) graphed from SOR until the time that 4 of the animals in each group had reached exhaustion. * significantly different ($p<.01$) by repeated measures ANOVA by group.

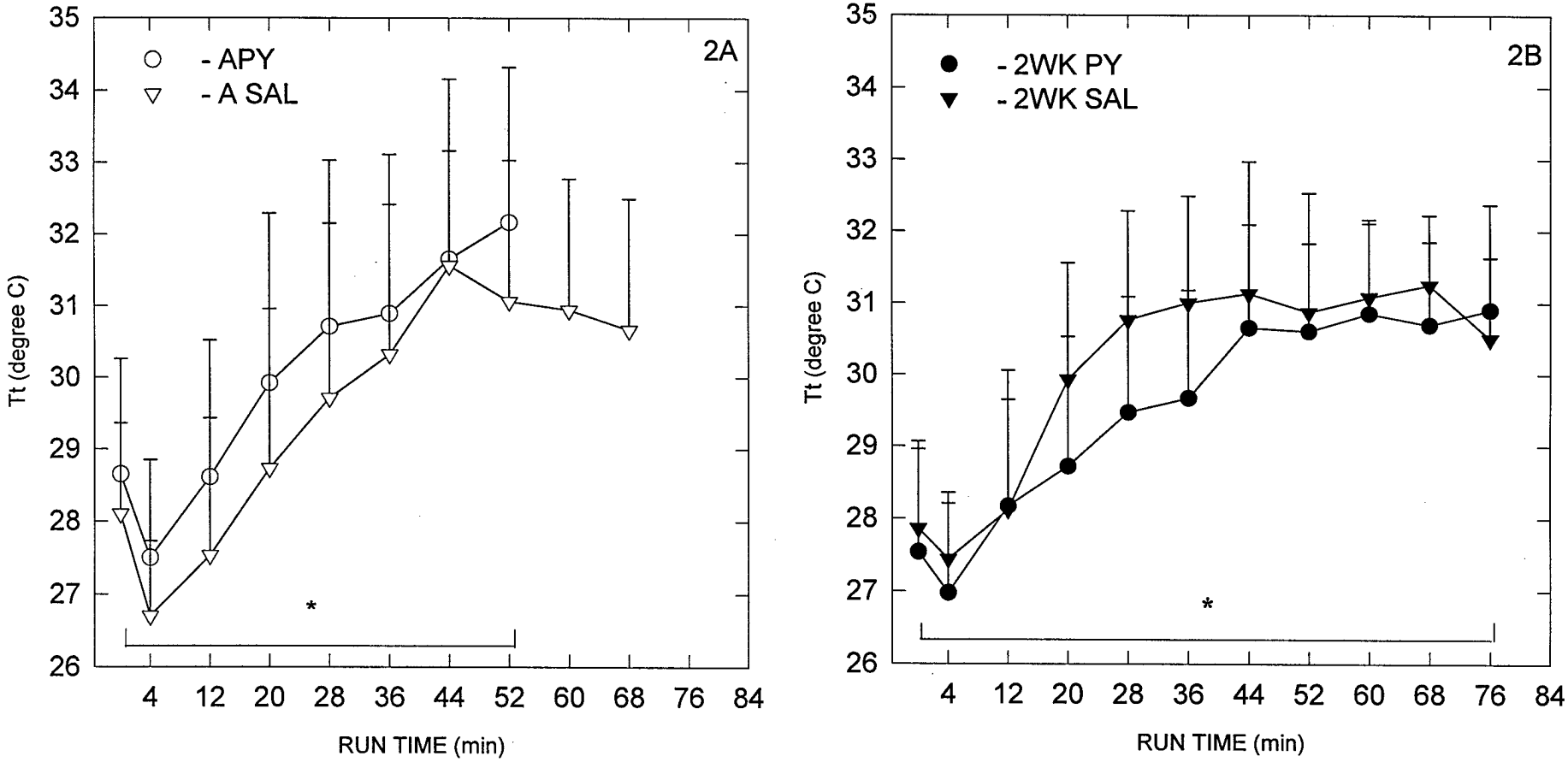

Fig. 2- Tt of ASAL vs APY (Fig 2A) and SAL2 vs PY2 (Fig 2B) graphed from SOR until the time that 4 of the animals in each group had reached exhaustion. * significantly different ($p<.01$) by repeated measures ANOVA by group.

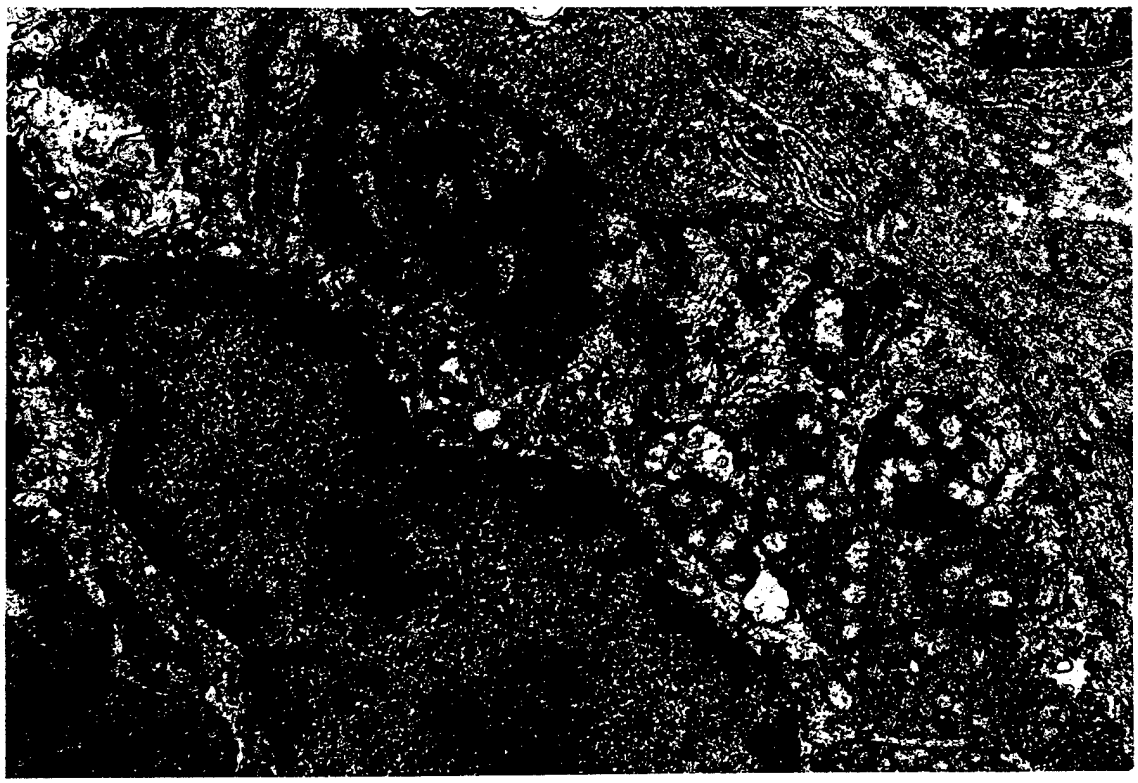

Fig. 3 Typical mitochondrial lesions produced at NMJs, in diaphragm muscle cells by high acute doses of PY (positive control, 1mg/kg), 20,100x.

Fig. 4 Typical mitochondria at NMJs in normal diaphragm muscle (negative control), 51,000x

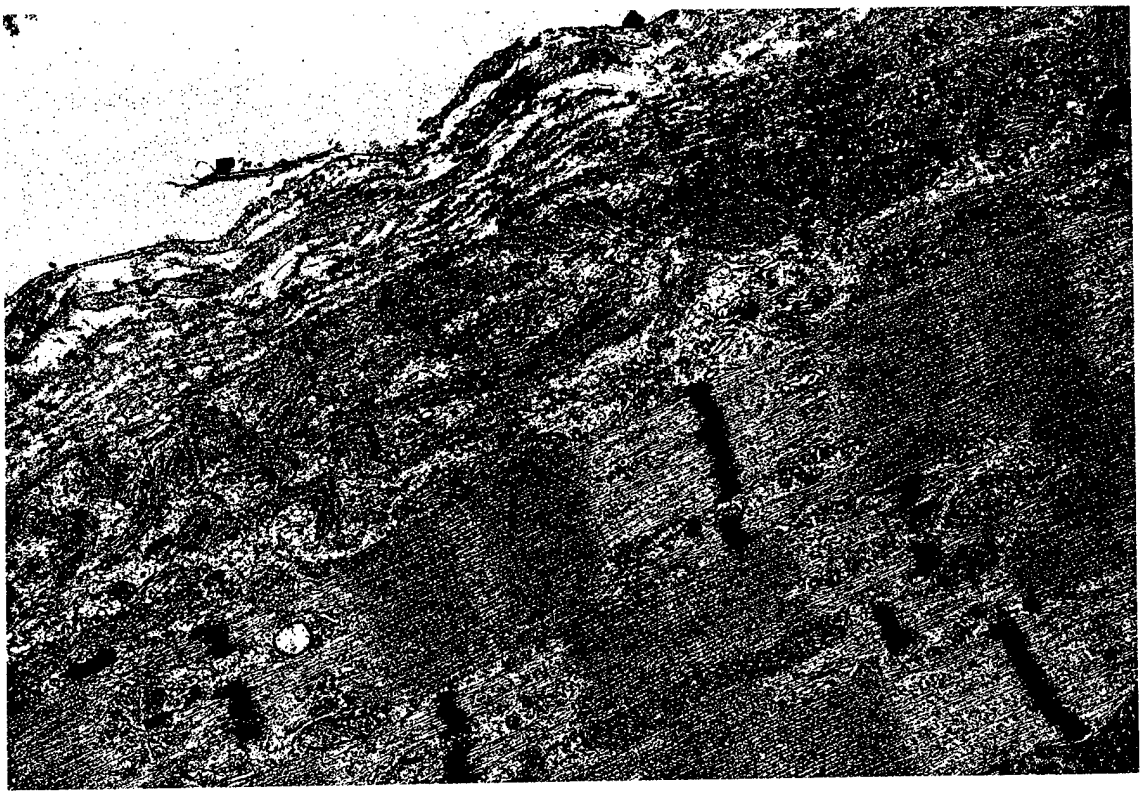

Fig. 5 Normal mitochondria in soleus muscle of rat after acute exposure to PY (APY, 100μg/kg) and run to exhaustion, 20,100x.

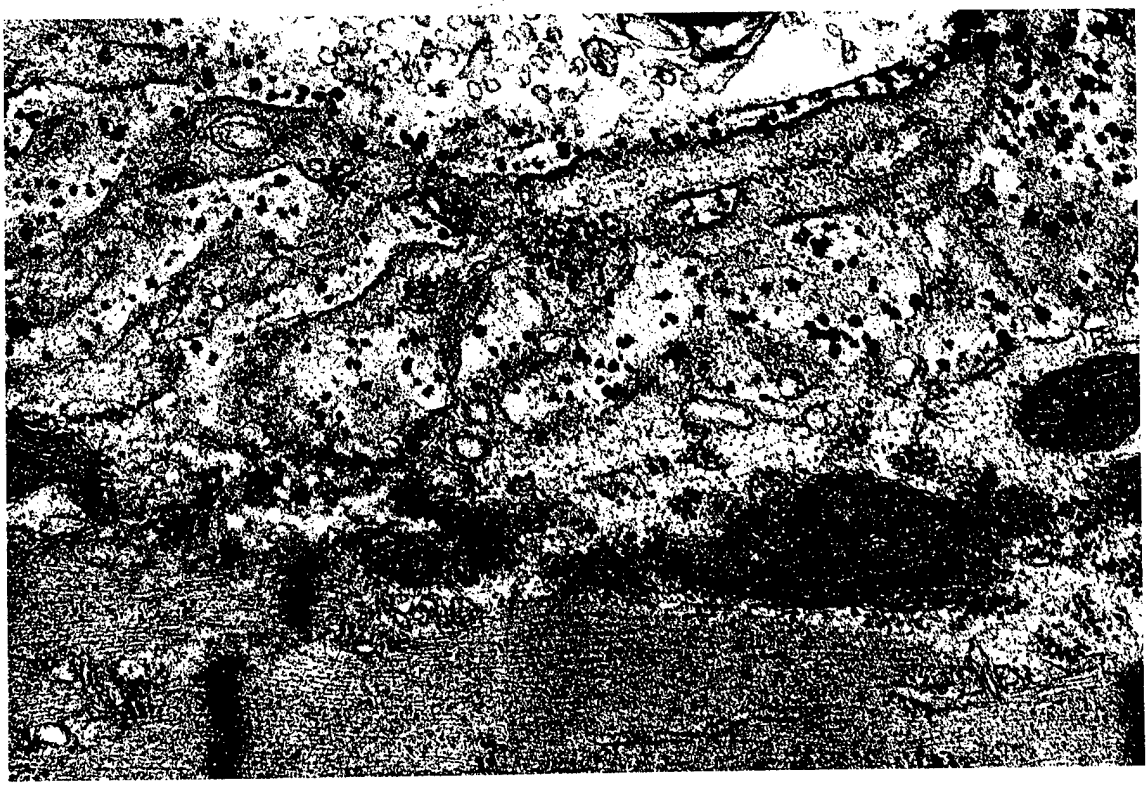

Fig. 6 Normal mitochondria in soleus muscle of rat after four week PY and run to exhaustion. Stain deposits (dark circles) used to localize NMJs are evident in this micrograph, 51,000x.

Fig. 7 Normal mitochondria in diaphragm muscle of rat after a four week exposure (PY4, 20μg/hr) and run to exhaustion, 24,900x.

Table 2. Exercise to Exhaustion

GROUP	RUN TIME (MIN)	HEAT RATE[a] (°C/MIN)	Tc SOR[b] (°C)	Tc EOR[c] (°C)	Tt SOR[d] (°C)	Tt EOR[e] (°C)
ASAL	71.7 ±16.7	.050 ±.016	38.50 ±.55	41.85 ±.37	28.10 ±1.26	30.55 ±1.68
APY	56.2* ±18.0	.052 ±.023	38.91* ±.25	41.54 ±.39	28.59 ±1.64	30.85 ±2.03
SAL2	74.2 ±24.3	.047 ±.015	38.55 ±.51	41.81 ±.34	27.86 ±1.10	29.69 ±1.36
PY2	76.7 ±22.4	.040 ±.016	38.47 ±.30	41.34* ±.50	27.31 ±1.64	30.47 ±1.14
PY3	68.5 ±25.1	.064 ±.026	38.14 ±.54	42.05 ±.33	27.97 ±1.36	29.63 ±1.79
SAL4	82.1 ±35.8	.052 ±.022	38.01 ±.20	41.65 ±.57	26.81 ±1.16	30.86 ±1.56
PY4	71.1 ±13.8	.048 ±.012	38.48* ±.53	41.76 ±.45	27.33 ±1.79	29.83 ±1.96

Values are mean ± SD.
* Significantly $p<.05$ different from the value directly above.
a Rate of rise of Tc during the run.
b Tc at start of run.
c Tc at the end of run.
d Tt at the start of run.
e Tt at the end of run.

Table 3. Core Temperature During Exercise

GROUP		0 min	4	12	20	28	36	44	52	60	68	76
ASAL	X	38.5	39.0	39.8	40.4	40.7	41.1	41.2	41.4	41.4	41.5	
	SD	0.6	0.6	0.6	0.5	0.5	0.5	0.4	0.5	0.4	0.4	
	N[a]	12	12	12	12	12	12	12	12	10	8	
APY	X	38.9*	39.4	40.1	40.7	40.9	41.4	41.1	41.2			
	SD	0.2	0.3	0.4	0.4	0.4	0.4	0.3	0.4			
	N	12	12	12	12	12	12	9	8			
SAL2	X	38.6	39.2	39.9	40.5	40.6	40.6	40.8	41.1	41.2	41.4	41.7
	SD	0.5	0.5	0.6	0.7	0.6	0.6	0.6	0.6	0.5	0.4	0.5
	N	12	12	12	12	12	12	10	10	9	9	8
PY2	X	38.5	38.9	39.5	40.0	40.3	40.6	40.5	40.8	40.9	41.1	41.2*
	SD	0.3	0.4	0.4	0.6	0.6	0.6	0.6	0.5	0.4	0.4	0.3
	N	11	11	11	11	11	11	11	11	9	7	7
PY3	X	38.1	38.6	39.9	40.7	41.0	41.4	41.4	41.6	41.8		
	SD	0.5	0.7	0.6	0.6	0.5	0.5	0.4	0.3	0.4		
	N	12	12	12	12	12	12	11	11	10		
SAL4	X	38.0	38.5	39.4	40.0	40.3	40.7	40.6	41.00	41.1	41.3	
	SD	0.2	0.2	0.4	0.5	0.6	0.7	.7	0.7	0.6	0.7	
	N	11	11	11	11	11	11	11	11	10	8	
PY4	X	38.4*	39.0*	39.8*	40.4*	40.6	41.0	41.1	41.3	41.5	41.7	
	SD	0.5	0.5	0.5	0.3	0.3	0.5	0.3	0.3	0.4	0.5	
	N	12	12	12	12	12	12	11	10	10	9	

a The number of animals in each group that were still running at that time.
* Significantly ($p<.05$) different from the corresponding SAL group.

Table 4. Tail Temperature During Exercise

GROUP		0 min	4	12	20	28	36	44	52	60	68	76
ASAL	X	28.1	26.7	27.5	28.7	29.7	30.3	31.6	31.1	30.9	30.7	
	SD	1.3	1.0	1.9	2.2	2.4	2.1	1.6	2.0	1.8	1.8	
	N[a]	12	12	12	12	12	12	12	12	11	8	
APY	X	28.6	27.5	28.6	29.8	30.7	30.9	31.6	32.2			
	SD	1.6	1.4	1.9	2.4	2.3	2.2	2.5	2.2			
	N	12	12	12	12	12	12	12	12			
SAL2	X	27.9	27.4	28.1	29.9	30.8	31.0	31.1	30.9	31.1	31.2	30.5
	SD	1.1	0.9	1.5	1.6	1.5	1.5	1.8	1.7	1.1	0.6	1.1
	N	12	12	12	12	12	12	10	10	9	8	8
PY2	X	27.3	26.8	28.2	28.7	29.5*	29.7*	30.6	30.6	30.8	30.7	30.9
	SD	1.6	1.3	1.8	1.8	1.6	1.5	1.4	1.2	1.3	1.5	1.5
	N	11	10	11	11	11	11	11	11	9	7	7
PY3	X	28.0	26.8	27.4	28.1	29.2	30.2	31.6	31.1	31.1		
	SD	1.3	1.2	0.9	0.9	2.1	2.6	2.4	2.0	2.2		
	N	12	11	11	11	12	12	11	11	10		
SAL4	X	26.8	26.2	28.3	28.5	29.3	30.1	30.7	30.3	30.2	30.9	
	SD	1.2	0.9	1.6	1.7	1.6	1.3	1.5	1.6	1.4	1.6	
	N	11	11	11	11	11	11	11	11	10	8	
PY4	X	27.3	26.5	28.0	28.8	29.6	30.2	31.3	30.2	30.3	30.4	
	SD	1.8	1.8	2.5	2.4	2.0	2.2	2.2	2.1	1.8	1.2	
	N	12	11	12	12	12	12	11	10	10	9	

a The number of animals in each group that were still running at that time.
* Significantly ($p<.05$) different from the corresponding SAL group.

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