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FINAL REPORT

PROJECT NO. US-1062-86

Breeding Fast Growing Red Tilapias with Improved Cold Tolerance by Introgression and Selection

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1991

A.

Final Scientific Report (III)

BINATIONAL AGRICULTURAL RESEARCH AND DEVELOPMENT FUND

February 1991

BARD

P.O. BOX 6

Bet Dagan, Israel

BARD project No. US-1062-86

Projects starting date: August, 1987

BREEDING FAST GROWING RED TILAPIAS WITH IMPROVED COLD
TOLERANCE BY INTROGRESSION AND SELECTION

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BREEDING FAST GROWING RED TILAPIAS WITH IMPROVED COLD TOLERANCE BY INTROGRESSION AND SELECTION

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BREEDING FAST GROWING RED TILAPIAS WITH IMPROVED COLD TOLERANCE BY INTROGRESSION AND SELECTION

ABSTRACT

Standardized short-term (3 to 12 hours) and long-term (8 to 12 days) cold tolerance tests were conducted to quantify phenotypic responses in tilapias (*Oreochromis* spp.). Genetic background and acclimation history were both significant factors affecting phenotypic response ($P < 0.05$), while body weight of fingerlings (within populations) was only weakly correlated, or not correlated with cold tolerance. Within populations, neither gender or color phenotype were significant factors influencing cold tolerance ($P > 0.05$). Rank-order of cold tolerance means among species was identical for short- and long-term tests: *Q. aureus* was most cold tolerant followed by *Q. niloticus*, *Q. mossambicus*, and *Q. hornorum*. Cold tolerance means for hybrid populations were intermediate to their respective parental means indicating no overdominance. Repeatability tests (cold tolerance tests repeated on the same individuals over time) provided data suggesting that cold tolerance is a heritable trait.

Among full-sib families (*Q. aureus*), 39 percent of phenotypic variation for cold tolerance was accounted for among family means, while the major reservoir of variation was among individuals within families.

Based on one generation of bidirectional selection for cold tolerance (individual selection), realized heritability estimates were calculated according to the ratio response/selection differential. Estimates for up-selected populations (most cold tolerant) were: *Q. aureus*, 0.33; *Q. niloticus*, -0.05; and F-3 hybrid, 0.31. Estimates for two down-selected populations (least cold-tolerant) were: *Q. aureus*, 0.04; and *Q. niloticus* 0.42. Based on these initial estimates, it should be possible to increase cold tolerance in *Q. aureus* and related hybrids via directed selection.

Companion studies were undertaken to evaluate correlated responses to bi-directional cold-tolerance selection on growth at suboptimal temperatures. Fingerlings from up-selected populations grew significantly faster ($P < 0.05$), than fingerlings from down-selected populations at temperatures ranging from 17.2° C to 21.2° C. Irrespective of selection criteria, growth rate of *Q. aureus* fingerlings under low temperature regimes was greater than *Q. niloticus* ($P < 0.05$).

Two-dimensional electrophoresis was applied to liver homogenates and blood plasma of selected tilapia populations and their respective controls to search for "cold tolerance proteins". Novel proteins of low molecular weight were detected in blood serum and liver tissue of several of the selected populations which had been acclimated to low temperatures. Identities and physiological functions of these proteins remain to be determined.

INTRODUCTION

Increasing tilapia (*Oreochromis* spp.) production in Israel over its present level (3000 t/year), or introducing their culture into the warm water aquaculture industry of the United States, is hampered by their sensitivity to winter temperatures and, in certain instances, low market acceptability. Growth and reproduction of commercial species are negligible at 20 and 22° C respectively (Behrends and Smitherman 1983, Soderberg 1990) and species specific mortality ensues after prolonged exposure to temperatures of 10 to 15° C (Wohlfarth and Hulata 1983).

Red tilapias, grown commercially in Taiwan largely for export to Japan and Singapore, are more attractive than "normal"- colored tilapias and command a premium price. However, many commercial strains of red tilapias grow slowly, suffer high mortalities and are more sensitive to low temperatures than other commercial strains of normal-colored tilapias (Behrends et al. 1982, El-Gamal et al. 1989).

Fast growing, cold tolerant hybrid strains of red tilapias may be developed via interspecific hybridization and introgression coupled with directed selection. This requires use of two breeding schemes: introgression of the red hereditary material into commercial strains of wild-type colored tilapias, and mass selection for improved cold tolerance within specific populations, or in segregating F₂ + generations. Introgression of desirable traits present in otherwise unproductive strains, into valuable commercial strains lacking these traits, is an established breeding practice. Improving cold tolerance in warm water fishes via directed selection has not been previously investigated (Gedrem 1988). It requires establishing adequate selection techniques and should yield first estimates of genetic parameters of this trait in different tilapia species.

Objectives of the research included: 1) development and evaluation of techniques for quantifying phenotypic variation for cold tolerance among species and interspecific hybrids, 2) estimation of heritability estimates for cold tolerance based on repeatability studies and/or mass (individual) selection, 3) development of commercial strains with both the desirable red coloration and improved cold tolerance, and 4) evaluation of two-dimensional (2-D) electrophoresis as a method for detecting novel cold tolerance proteins in strains of tilapia selected for cold tolerance.

MATERIALS and METHODS

Fish stocks

Fingerling stocks of four species: *O. aureus* (Israel), *O. niloticus* (Egypt, Ghana), *O. mossambicus* (South Africa) and *O. hornorum* (Tanzania), were evaluated in both short- and long-term cold tolerance studies. Several hybrids and synthetic strains (introgressed populations) were also evaluated for comparative purposes and to gather preliminary information on probable gene action, i.e., additive, dominance, and/or overdominance. In tests conducted in the U.S., fingerlings tested were randomly selected from populations derived from multiple spawns encompassing 10 to 40 families.

Red and normally pigmented hybrid strains of *O. aureus* and *O. niloticus* used in several of the preliminary studies were produced over several years via introgressive hybridization (Behrends and Smitherman 1990). This entailed, for example, producing F-1 hybrids by crossing *O. aureus* females with a red hybrid (predominantly *O. mossambicus*) and subsequently backcrossing red hybrid males to *O. aureus* females for multiple generations. During this same period, an analogous strain was produced by crossing *O. niloticus* and the red hybrid (predominantly *O. mossambicus*), followed by multiple generations of backcrossing to *O. niloticus*.

For purposes of clarity, interspecific crosses will be denoted accordingly: (initial of female species x initial of male species); e.g. (mxa) corresponds to the cross female *mossambicus* x male *aureus*. Backcross generations will be denoted by a subscript, e.g., b-5, a fifth generation backcross.

Phenotypic response to rapid cooling: variation within and among populations

Several rapid-cooling cold tolerance studies, each with a duration of 3 to 12 hours, were conducted to evaluate the impact of absolute temperature decline, rate of temperature decline, and rate of temperature gain (recovery phase of experiment), on survival and rate of recovery from cold narcosis. Individually or together, these parameters can provide estimates of cold tolerance.

In U.S. studies, stocks of fingerlings used in short-term studies had similar thermal histories, i.e., all were slowly acclimated to 15-16°C and held for at least 2 weeks prior to testing. Feeding practices used during acclimation included twice/day ad-libitum feedings, seven days/week. Diets were pelleted rations containing 32 to 42 percent crude protein (Purina Inc., St. Louis, Mo.). Water management during acclimation was flow-through of ground water.

Tilapia fry (< 1g), or fingerlings ranging from 2.5 to 85 grams were stocked at densities of 5 to 10 fish/replicate 10-l aquaria. Each genetic group was replicated 3 to 5 times. In studies where different genetic groups were stocked communally, individuals were cold-branded (Myers and Iwamoto et al. 1986), to facilitate group identification.

Commercial chiller units (Frigid Units, Toledo, Ohio, U.S.A.), and cubed ice were used to decrease temperature of recirculated water from standard acclimation temperatures of 15 to 16°C to a low of 4 to 6°C at a rate of 1.5 to 3.5°C/hour. Chilled water was pumped from the chiller tank to an overhead 400 l. reservoir which supplied aquaria, via gravity, with well-mixed water. Flow rate to each aquarium was calibrated with ball-type valves to provide a 1.25-5.00 minute retention time. Water exiting aquaria was directed back to the chiller tank. Fish were not fed during cold tolerance testing periods.

During tests, temperature was recorded at 5 minute intervals. A three-man team observed and recorded fish behavior. When all test fish lost consciousness due to cold narcosis (no response to external stimuli), chiller units were shut-off and timers reset to zero. Subsequently, a flow of 2 to 6 l/min. of 16°C water was directed into the chiller tank and mixed. These flow rates were sufficient to warm the recirculated water at an average rate of 2.7 to 3.0°C/hour.

Cold tolerance was quantified by recording the time interval from time zero to recovery from cold narcosis. Recovery was defined as regaining consciousness and attaining and maintaining an upright position. General linear models procedure (Barr et al. 1979) was used to analyze data and detect differences among populations for the parameter mean time to recovery from cold narcosis. The randomized complete designs included fixed effects due to genetic group and color phenotype. Random effects included aquaria, and individual fish within group within aquaria. Simple correlations were calculated, by population, to quantify the relationship between individual weight and time to recovery.

Phenotypic response to rapid cooling: impact of interim temperature and recovery period on repeatability

When repeated measurements are made on the same trait of several individuals within a population, it is possible to calculate a liberal estimate of genotypic heritability (Becker 1984). This can provide broad-sense heritability estimates prior to performing classical multiple-generation breeding studies.

In preliminary tests conducted in 1988, four random samples of 10 fingerlings each (F-1 hybrid *O. niloticus* x *O. aureus*) were subjected to rapid cooling. Using ice chips, water temperature was reduced from 17°C to 5.5°C in three hours at which time all fish were unconscious. Water temperatures were then slowly increased with the aid of warm water. Temperature at loss of consciousness and temperature at time of recovery were recorded for individual fish. The first ten individuals recovering at temperatures between 10 to 10.5°C were designated as cold tolerant selects. Both the selected and non-selected fish were kept at 16°C for seven days at which time a second repeat test was conducted.

Fingerlings tested in the repeat series consisted of the selected stock, two non-selected samples from the previous test and two further samples of fish of the same stock, but which had not previously been

exposed to low temperatures. In the repeat cooling test, water temperature was again reduced from 15 to 5°C, at which time all fish had lost consciousness. An additional four rapid cooling tests were conducted in which the time interval between initial and repeat cooling was varied from 17 to 46 days. During these interim recovery periods, water temperature was raised to 26°C and the fish were fed 25 percent protein pellets.

Phenotypic response to rapid cooling: cold tolerance selection of O. niloticus, Ghana strain

Two rapid cooling tests were conducted in 1988 using fingerlings of O. niloticus (Ghana), the strain most commonly used as dams in Israeli aquaculture. Temperature management was similar to that used in evaluating hybrid fingerlings (see previous page). Tests were carried out in five aquaria, each stocked with 40 or 55 fry. Temperatures at loss of consciousness and recovery were recorded for individual fish. The first twenty percent to regain consciousness were designated up-selects and retained as broodstock. Those regaining consciousness at higher temperatures were also retained as "non-selected" broodstock. Both stocks were assortatively mated; fingerlings from these matings were subsequently evaluated for cold tolerance (survival) in three slow-cooling tests.

Phenotypic response to slow cooling: variation within and among populations

Long-term cold tolerance studies were conducted in closed-cycle recirculating unit similar to, but larger than, the rapid cooling system discussed earlier. Water cooling was provided by two 1-horsepower cooling units (Frigid Units, Toledo, Ohio), and cubed ice. A residential air conditioner was operated to remove residual heat from the cold tolerance room.

Tilapia fingerlings, ranging in weight from 2 to 50 g, were stocked into 90 l aquaria at a rate of 150/aquarium. Each of the genetically distinct populations was replicated three to five times. All groups had been acclimated to 16°C 2 weeks prior to the study. In several studies, the fingerlings were cold branded to facilitate stocking of several genetic groups (or families), into each of the replicate aquaria. Communal stocking can provide enhanced statistical power by increasing error degrees of freedom, and thus a more precise measurement of the genetic contribution.

In the U.S., water temperature was decreased from acclimation temperature (16°C) to 5°C at a rate of 1°C/day. This procedure reputedly allows temporal physiological adaptation (no temperature shock), and provides good estimates of mean lower lethal temperature limits (Shafland and Pestrak 1982). In Israeli tests, temperature was decreased 2°C and then increased 1°C within a 24 hour period to more closely mimic seasonal ambient cooling.

In the U.S., time (to the nearest hour) and temperature (to the nearest 0.1°C) were recorded for each fish at loss of equilibrium, loss of consciousness, and death, i.e., cessation of opercular movements. Post-mortem data collection included individual weight, determination of sex via gonadal squash (Guerrero 1975), and incidence of fungal infection by Saprolegnia spp.

Mean lower lethal temperatures (MLLT), and mean cumulative degree hours (MCDH) to an unconscious state or death were calculated for each population. MCDH is calculated as the sum total of degree hours below a set threshold (acclimation) temperature, e.g. 16°C. For example, a fish held for one hour at 14°C, would accumulate 2 degree hours if the threshold temperature was 16°C; if held an additional hour at 13°C, the sum would be (16-14) + (16-13), or 5 degree hours, etc. Thus, progressively lower temperatures contributed a progressively larger number of degree hours to the sum.

General linear models procedure (Barr et al. 1979) was used to detect differences among populations for the parameters MLLT and MCDH. The randomized complete design included fixed effects due to population, sex and color phenotype. Random effects included aquaria and individual fish within group within aquaria. Separate analysis were run for each color phenotype since both red- and normal-colored phenotypes were costocked into replicate aquaria, thus confounding effects due to color phenotype and

genetic group. Within populations, simple correlation was used to quantify the relationship between individual weight and mean cumulative degree hours.

Phenotypic response to slow cooling: variation within and among full-sib families of O. aureus

Twenty full-sib families of Oreochromis aureus, comprised of 18 to 52 individuals per family, were subjected to a long-term cold-tolerance test. Six to 10 fingerlings from each family were costocked into each of five replicate aquaria. Each family was cold branded with a distinct mark which allowed costocking of families.

Water temperature was decreased from an acclimation base temperature of 15°C to 5°C at a rate of 1°C/day. Temperatures were recorded manually on-the-hour for the duration of the experiment. As individual fish lost equilibrium due to cold narcosis, they were removed from the experiment and slowly reacclimated to 16°C for later use in selective breeding trials. Both elapsed time and temperature at loss-of-equilibrium were recorded for individual fish. Data were analyzed using a general linear model to partition phenotypic variance into components related to differences among and within families.

Phenotypic response to slow cooling: survival of O. niloticus (Ghana strain) fingerlings previously selected by the rapid cooling method

Ghana strain fingerlings selected for cold tolerance via the rapid cooling method (see page 4), were subsequently grown to maturity, and mated assortatively to produce fingerlings of up- selected parents and non-selected parents. Fingerlings were slowly acclimated to 16°C over a period of 12 hours and held at that temperature for six to ten days prior to starting the slow- cooling tests. Acclimation and the slow-cooling tests were conducted in large custom designed cooling units, complete with a programmable controlled switchboard (see Plate 1a-c). The units are located at the Dor, Israel Fish and Aquaculture Research Station.

Experimental design for each of the three slow-cooling tests included evaluation of sixty fingerlings per select group, consisting of three replicates of twenty fingerlings each. Temperature management (Figure 2), included a diurnal pattern of decreasing temperature 2°C followed by an increase of 1°C until temperatures of 9-10°C were reached. Subsequently, temperature was stabilized or slowly increased. Survival of test fingerlings was plotted as a function of temperature and elapsed time.

Bidirectional selection for cold tolerance in three populations of tilapia: realized heritability estimates

Three populations of tilapia (O. aureus, O. niloticus, and a red F-2 hybrid strain), consisting of 600 fingerlings per population, were subjected to a standardized cold-tolerance test. Water temperature was decreased from 16°C to 5°C at a mean rate of 1°C/day. Selection criteria was based on time and temperature at loss of consciousness due to cold narcosis. Within populations, bidirectional selection was accomplished by selecting the most cold tolerant, i.e. those to lose consciousness last (upper 10 percent) and the least cold tolerant, i.e. those to lose consciousness first (lower 10 percent).

Fifty to sixty broodstock from each of the selected populations (up- and down-selected), were assortatively mated in replicate earthen ponds. Proper control populations were created by respawning the original parental lines. Resulting offspring, consisting of several thousand of each population, were cultured for approximately three months, harvested in the fall, and overwintered at 16°C. Two-hundred fingerlings from each population were subsequently cold-branded, stocked communally into replicate aquaria, and subjected to a standardized cold tolerance test.

A second generation of bidirectional selection was also completed during the spring of 1990. Selection was accomplished by retaining the upper 50 percent (100 most cold tolerant), and the lower 50 percent (100 least cold tolerant) of the selected fingerlings from the first generation selection trial. Second generation selects, and respective control populations, were assortatively mated, fingerlings produced, overwintered, freeze branded, and subsequently subjected to a standardized slow-cooling cold tolerance

a

Plate 1

b

c

Figure 2. Temperature management for slow cooling tests included a diurnal pattern of 2° C decrease followed by a 1° C increase. Dor, Isreal.

test. Realized heritability estimates for the first and second generation of selection were calculated based on the ratio response to selection differential (Falconer 1981). Standard errors of the estimates were calculated for only the first generation of selection, due to limitations of the formula (Prout 1962).

Bi-directional selection for cold tolerance in tilapias: correlated responses in growth at suboptimum temperatures

Two generations of bidirectional selection for cold tolerance were conducted on populations of *O. aureus* and *O. niloticus* (Egypt strain). Selection criteria were summarized in a previous section on heritability estimates. Fingerlings in this study were generated from mass spawns encompassing 20 to 30 pair of broodstock per treatment group. Fingerlings were mechanically graded to a mean weight of approximately 2g to eliminate, as much as possible, differences in initial weight. Fingerlings were randomly allocated by group to aquaria and cultured for three contiguous 30 day trials at mean suboptimum temperatures of 17.2°C, 19.2°C, and 21.2°C respectively. Each temperature-group treatment was replicated 3 times with 20 fingerlings per replicate.

All treatments received a salmon starter diet (50 percent crude protein) at 3 percent of body weight in equal a.m. and p.m. feedings. Feed rates were adjusted biweekly based on the average biomass of the fastest growing group. Water management was pass-through with a retention time of 5 minutes. Thus, with the exception of water temperature, water quality was near optimum for realized growth potential.

Fingerlings were weighed individually to the nearest 0.1g at two week intervals. Two-way analysis of variance, with factorial arrangement, was used to detect differences in growth within 30 day trials. Main effects were species (*aureus* vs *niloticus*), and selection criteria (up-select vs down-select vs control). Where warranted, covariance was used to adjust final mean weights by initial weights, a statistical practice used to compensate for differences in initial weight.

Two-dimensional electrophoresis: evaluation of selected strains of tilapia, University of Alabama, USA, Birmingham campus.

The electrophoretic work was conducted on a subcontract basis between TVA and the University of Alabama. Dr. George Cline directed this area of research. Blood plasma and liver tissue samples from tilapias were provided weekly by TVA for analysis. Each sample was coded with a TVA I.D. number. Plasma samples arrived in heparinized capillary tubes; liver samples on wet ice or frozen. Three shipments by overnight air express arrived 2 or more hours late and were at ambient temperature.

Blood samples were centrifuged for 20 minutes in a standard clinical capillary centrifuge and hematocrits and plasma volumes recorded. The capillaries were nicked with a triangular file and the plasma portion broken off and transferred to a 600 microliter microfuge tube. Samples were prepared for electrophoresis either of two ways. The first protocol used 1 part plasma with 2 parts of a solubilizing mixture which contained 2 percent sodium dodecylsulfate (SDS), 5 percent 2-mercapto-ethanol (2-ME) and 10 percent glycerol. The mixture was heat denatured at 95 °C for 5 minutes, quickly cooled and saturated with urea before electrophoresis.

The second protocol for plasma started with mixing 1 part plasma to 1 part stock glycerol. (Some samples were stored at - 80°C at this point.) Next, 5 parts of glycerated sample were mixed with 1 part of a solubilizing mixture which contained: 0.1 ml stock SDS, 0.02 ml pH 3-10 ampholytes, 0.18 ml pH 5-7 ampholytes, 0.1 ml 2-mercaptoethanol and 0.2 ml of NP-40, a non-ionic detergent. After mixing, the solution was saturated with urea before analysis. Experiments to find the best detergent/protein dispersant for tilapia plasma proteins involved substituting Triton-X 100, the original agent in the sample mix and the gels, with other agents. Agents tried in various micelle concentrations and with and without urea were: Nonidet-40 (NP-40), CHAPS, Zwitteragents 16, 14 and 12.

Liver samples were prepared by first weighing the liver, then dicing it with scissors, then homogenizing it in a 1.5 ml microfuge tube with a rotary tight-fitting pestle in an homogenizing solution. The solution

was 9 molar with urea, 4 percent NP-40, 2 percent ampholines and 2 percent 2-ME. Five mg homogenizing solution was used for each mg of liver (i.e., a 5:1 dilution). The brei was centrifuged at 16,500 x G for 15 minutes to clarify the supernatant. The supernatant was divided into aliquots in 0.6 ml plastic microfuge tubes and frozen if not used that day. From 5 to 15 microliter sample volumes were used per IEF tube gel in the first part of the 2-D separation.

Experiments to find the best detergent/protein dispersant for liver tissue involved setting up a series of second dimension gels (in duplicate) which contained either Triton-X 100, NP-40, CHAPS, and Zwitteragents 12, 14 or 16.

The first dimension separations of both plasma and liver samples were made in 1 mm capillary tubes in which was cast a 4 percent acrylamide gel, 5.4 percent cross-linked. The gel also contained 9.5 M urea, 2 percent NP-40, 5 percent 2-ME, 1.6 percent ph 5-7 (or 4-6.5) ampholyte and 0.4 percent ph 3-10 ampholyte.

First dimension separations used de-gassed 20 mM NaOH as the catholyte and de-gassed 10 mM phosphoric acid as the anolyte. Sample sizes were normally 5 microliters for plasma and 15 microliters for liver. Up to 16 individual tube gels were electrofocused at the same time and were run at 500 volts for 10 minutes and 750 volts for 3.5 hours. Focused gels were either equilibrated (cold denatured) for 10 minutes in reducing buffer (0.06 M Tris, pH 6.8, 2.3 percent SDS, 5 percent 2-ME, 10 percent glycerol and 0.01 percent bromphenol blue) and layered onto a separating gel for the second dimensional separation or, stored in 0.25 ml of reducing buffer in a 0.6 ml capped centrifuge tube at -80C. In most cases, 4 tube gels were run in parallel.

Tube gels were fused to the top stacker gel portion of separating gels with a melted 1 percent agar solution which contained pH 6.8 stacking gel buffer, 2.5 percent SDS, 5 percent 2-ME, 8 percent glycerol, and 0.01 percent bromphenol blue dye. The gel holders were warmed to 60 C first in an oven, 0.5 ml of the fusing solution was carefully layered onto the stacking gel and the tube gel positioned in contact with the stacking gel, acidic end to the left and without trapped bubbles. The agar was gelled quickly and the IEF tube gel locked into place by rubbing one side of the glass plate with an ice cube.

Acrylamide gels for second dimension separations were 7 percent at 2.67 percent X-L for the plasma samples and 12 percent at 2.67 percent X-L for the liver samples and were run in the BioRad mini-PROTEAN II dual slab cell. Both types of 1 mm thick gels contained 0.375 M Tris at pH 8.8 and had overlaying stacker gels of approximately 5 mm height. The stacker gels were 4 percent acrylamide, 0.125 M Tris at pH 6.8. The electrode (running) buffer was Tris-glycine at pH 8.3 and separating time was approximately 35 minutes at 160 volts. Each gel had a molecular weight standard run along the right edge.

Separating gels were removed from the holders and immediately "fixed" in a 40 percent methanol-10 percent acetic acid solution. Gels were then stained by the BioRad silver stain method. Early silver stained gels were double stained with Coomassie Blue R-250 dye. Some silver stained 2-d gels were scanned with an LKB/Pharmacia laser scanner and the scans saved by computer. Upgraded software for 2-D scanning and processing was delivered late in the grant program and was of little use due to irregularly sized gels (due principally to shrinking and swelling). Gels patterns were recorded with photography.

RESULTS

Phenotypic response to rapid cooling: variation within and among populations

Mean survival and recovery time from cold narcosis was a function of genetic group and environmental components, including absolute temperature change, and rate of temperature change (Tables 1-3). Under similar temperature regimes (Trials 1-3, Table 1), survival and mean recovery times differed significantly among genetic groups ($P < 0.05$). Among species, *O. aureus* was most cold tolerant, requiring 43 to 48 minutes for recovery, followed by *O. niloticus* (69 minutes), and *O. mossambicus* (110 minutes). Mean survival also followed a similar trend: *aureus* *niloticus* *mossambicus* (Table 1).

Small changes in experimental protocol influenced results dramatically. Table 2 summarizes results of two trials, conducted simultaneously, in which the minimum critical temperature challenge differed by less than 1° C. Survival of *O. niloticus* dropped from 98 to 23 percent at 5.5° C and 4.6° C respectively, while mean recovery time increased from 58.7 to 90.2 minutes respectively (Table 2).

Mean survival and recovery times among hybrid and introgressed populations were always intermediate to or not significantly different from the parental types (Tables 1-3). Hybrids with close ancestry

Table 1. Mean recovery time (minutes) and survival (%) of tilapia fingerlings subjected to rapid cold tolerance test. Water temperature was decreased from 16°C (acclimation temperature) at 3.3-3.5°C until all fish were comatose and did not respond to external stimuli. Subsequently (time = 0), water was warmed at an average rate of 2.7°C/hour until surviving fish recovered and maintained an upright position.

Species/hybrid	n	Survival (%)	Mean recovery ¹ time (min.)	Coefficient variation (%)	Correlation (r) weight:recovery time
Trial 1					
<i>O. aureus</i>	30	90	43.9 a	16.0	-0.05 ($P > 0.79$)
<i>O. mossambicus</i>	30	47	109.6 c	11.6	-0.39 ($P > 0.16$)
Hybrid "pink" ²	30	63	70.1 b	20.6	-0.08 ($P > 0.74$)
Trial 2					
<i>O. aureus</i>	36	100	46.1 a	16.4	0.01 ($P > 0.91$)
F-1b (mxa)x(a)	36	100	60.5 b	27.5	-0.23 ($P > 0.17$)
F-2 (mxa)x(mxa)	36	78	72.0 b	31.8	-0.17 ($P > 0.38$)
Trial 3					
<i>O. aureus</i>	30	90	47.6 a	14.5	-0.05 ($P > 0.77$)
<i>O. niloticus</i>	30	83	69.3 b	12.8	0.01 ($P > 0.93$)
(mxa)x(pink)	30	83	62.2 b	15.9	-0.23 ($P > 0.26$)

¹ Means within a trial followed by a different letter are significantly different ($p < 0.05$), according to Student-Newman-Kuels Test.

² "Pink" hybrid derived from the cross (red *aureus*_{b2} x red *aureus*_{b2}; b2 refers to second generation backcross of red male to normally pigmented *aureus* female). Pink phenotype is homozygous with respect to gene coding for red coloration.

to *O. aureus*, e.g. F-1 backcross and introgressed populations, had mean recovery times proportional to their genetic contribution from *O. aureus*. Similar results were recorded for *O. niloticus* hybrids.

In general, interspecific populations were more variable than intraspecific populations with respect to recovery time (see coefficients of variation, Tables 1-3). The greatest amount of variation, 31.8 percent, was recorded for the F-2 population (mx_a) x (mx_a), followed closely by the F-1 backcross (mx_a) x (a), in which the C.V. was equal to 27.5 percent (Table 1). This follows theoretical expectations of increased phenotypic variance in F-2 and backcross hybrids due to segregation and recombination of novel genotypes. Phenotypic and genetic variation is generally greatest in hybrid populations in which the parental genotypes are divergent with respect to the trait being considered.

There were instances where results did not follow expectations: in trial 2 (Table 2), the within-population variation for *O. aureus* and the F-2 hybrid (ax_n) x (ax_n) were very low, i.e., C.V. = 3.1 and 4.6 percent respectively. This can be attributed to a suboptimum temperature challenge of 4.6°C followed by a rather rapid increase in temperature during the recovery phase. Better phenotypic resolution in *O. aureus* and its hybrids was accomplished with a lower temperature challenge (4°C), and a temperature recovery phase averaging approximately 2.7°C/hour (Table 3). Body weight of individual fingerlings within a genetic group was not a significant factor contributing to variation in individual recovery time ($r < 0.40$, $P > 0.15$; Table 1).

Table 2. Mean recovery time (minutes) of tilapia fingerlings subjected to rapid cold tolerance test. Water temperature was decreased from 16°C (acclimation temperature) to 5.5°C (Series 1) and 4.6°C (Series 2) respectively at a rate of 2.7°C/hour. Subsequently, water temperature was increased at approximately 3.0°C/hour until all surviving fish recovered and maintained an upright position.

Species/hybrid	n	Survival (%)	Mean recovery ¹ time (min).	Coefficient variation (%)
<u>Trial 1</u>				
<i>O. niloticus</i>	40	97.5	58.7 a	10.4
<i>O. mossambicus</i> (n)	40	82.5	85.1 b	14.5
<i>O. mossambicus</i> (r)	40	37.5	78.9 b	23.0
<i>O. mossambicus</i> (g)	40	0	-	-
<i>O. hornorum</i>	40	5.0	207.2 c	0.08
<u>Trial 2</u>				
<i>O. niloticus</i>	40	22.5	90.2 c	8.9
<i>O. aureus</i>	40	100.0	43.2 a	3.1
F-2 hybrid (ax _n) x (ax _n)	40	82.5	60.1 b	4.6
<i>O. mossambicus</i> (r)	40	0.0	-	-
<i>O. mossambicus</i> (g)	40	0.0	-	-

¹ Means within a trial followed by a different letter are significantly different ($p < 0.05$), according to Student-Newman-Kuels Test.

Three populations of red hybrids, each with red and normally pigmented phenotypes, were tested to quantify the effect of population, color phenotype within population, and related interactions. The red gene complex, originating from a mutant strain of *O. mossambicus*, was transferred via introgressive hybridization to each of the populations. ANOVA revealed no significant differences among phenotypes ($F=0.07$, $P>0.79$), and no significant population x phenotype interactions ($F=1.19$, $P>0.31$). Thus, phenotypes were pooled to evaluate differences among populations. ANOVA and means separation tests revealed the two reciprocal F-2 hybrids were not significantly different, but that both were significantly more cold tolerant than the red niloticus hybrid (Table 3). After five generations of backcross breeding (no selection), the red niloticus hybrid was nearly identical to the pure strain of *O. niloticus* with respect to cold tolerance.

Phenotypic response to rapid cooling: impact of interim temperature and recovery period on repeatability

In the initial rapid cooling test, all fish had lost consciousness at 5.5°C. In the recovery phase of the test, fingerlings began to regain equilibrium at 10°C. Fish recovering between 10°C and 10.5°C, a total of 10 fish from three aquaria, were selected for further tests (Table 4). However, fingerlings in the fourth aquaria did not regain equilibrium until temperature reached 11°C; these fish were excluded from the experiment. It was presumed that their later recovery was due to small unnoticed inaccuracies in the temperature control. Selection intensity in this experiment was 10 out of 30 fish.

Table 3. Mean recovery time (minutes) and survival (%) of tilapia fingerlings subjected to rapid cold tolerance test. Water temperature was decreased from 18° C (acclimation temperature) to 4.0° C at a rate of 1.5° C/hour, at which time all fish were comatose and did not respond to external stimuli. Subsequently, water temperature was increased at 2.7° C/hour until all surviving fish recovered and maintained an upright position.

Species/hybrid	n	Survival (%)	Mean recovery time (min.)*	Coefficient variation (%)
<i>O. aureus</i>	50	98	60.6 a	22.8
<i>O. niloticus</i>	50	18	134.7 c	22.0
Hybrid (red Nile) ¹	50	16	133.2 c	27.5
F-2 red hybrid ²	50	88	82.8 b	17.0
F-2 red hybrid ³	36	86	83.6 b	23.0

* Means followed by a different letter are significantly different ($p<0.05$), according to Student-Newman-Kuels Test.

1 Red hybrid derived from the cross (red niloticus_{b5} x red niloticus_{b5}), where b5 corresponds to the 5th generation backcross.

2 Red hybrid derived from the cross (red aureus_{b5} x red niloticus_{b5}) x (aureus x niloticus).

3 Red hybrid derived from the cross (red niloticus_{b5} x red aureus_{b5}) x (aureus x niloticus)

Table 4. Initial rapid cooling test (I), January 25, 1988.

Time	Temp (°C)	No. of fish* recovering in aquarium No.			
		1	2	3	4
9:00	17.0	Beginning of experiment			
11:50	5.5	All fish unconscious			
12:30	8.5				
12:37	10.0		3**		
12:40	9.5	3**			
12:58	10.5	2**	6	2**	
13:03	11.0				2
13:20	11.5	All	All but one	All but one	

* No. of fish per aquarium - 10

** Selected

In the repeat cooling test, fish which had previously participated in the cooling test, both selected and non-selected, appeared to be in a poor physical shape, especially noticeable from the condition of their caudal and other fins. Control fish did not suffer from this defect. Fish previously cooled started losing consciousness much earlier, i.e. at 10°C to 9°C than their controls (Table 5). Furthermore, when water temperatures were raised, practically all control fish recovered consciousness at 10°C, but fish previously cooled, selected or non-selected, did not all recover consciousness even at 14°C. Apparently those fish which were previously exposed to low temperatures and in poor physical condition due to acclimation history, exhibited increased sensitivity to low temperatures in the repeatability trial.

There was no real difference in cold tolerance between previously selected and non-selected fish when both groups were in poor condition. These results illustrate that tests involving repeated cooling require an intervening period for recovery from the effects of the first cold shock.

Table 5. Repeat rapid cooling test (II), February 1, 1988

Time	Temp (°C)	No. of fish recovering in aquarium No.				
		1 Selected* (8)	2 Non-selected* (14)	3 Non-selected* (14)	4 Control**	5 Control**
8:45	15.0	Beginning of experiment				
10:10	10.0	4 fish becoming unconscious				
10:20	9.0	3 fish becoming unconscious				
11:15	5.0	All fish unconscious				
12:10	8.5				1	
12:15	9.0				2	
12:21	9.0				1	
12:25	9.0					3
12:30	10.0				All but one	All
12:45	11.0	2	1			
12:58	11.5		1			
13:00	12.0		2	1		
13:10	12.5		1	3		
13:30	14.0	2	1			

* Fish subjected to cooling test on January 25

** Fish not previously cooled

An additional four rapid cooling tests were conducted in which the time interval between initial and repeat cooling was varied from 17 to 46 days. The results of these tests are summarized in Figures 3a-b and 4a-b. Recovery of consciousness in previously selected and non-selected fish started at 9°C. All previously selected fish had recovered at 10°C, but for the previously non-selected fish this occurred at 12°C. Control fish (i.e., those not previously subjected to cooling) showed intermediate rates of recovery over most of this range, though some control fish recovered consciousness only at 13°C. This demonstrates that fish selected in rapid cooling tests are more tolerant to low temperatures than random samples from the population. Also, an intervening period of 17 days between initial and repeated testing, during which temperature is raised and fish fed, is sufficient to recover from the physiological shock of exposure to low temperatures.

Phenotypic response to rapid cooling: cold tolerance selection of O. niloticus, Ghana strain

Acclimation temperature during this rapid cooling test was 15°C. All fish had lost consciousness by 7°C (except one replication in the second test, in which fish became unconscious at 6°C). Regaining of consciousness and equilibrium started at 8-9°C and by 12°C the required number of fish (about 20 percent in each aquarium) had recovered (see Table 6).

Note that fish did not begin to recover consciousness at the same temperatures in all replications. Particularly noticeable was aquarium no. 3, in which recovery began to take place at 12°C in both tests. Presumably this is an artifact, possibly due to insensitivity of the thermometer used in this aquarium. Similarly recovery required a higher temperature in this test, i.e. 20 percent of each sample had recovered by 12°C, whereas in previous tests over half the fish not previously exposed to low temperature (i.e. the control groups) had recovered at 10°C.

The fish in this series of tests were purebred O. niloticus fry, whereas in previous tests, hybrid fingerlings of O. niloticus x O. aureus had been used. The difference in tolerance to low temperatures

Table 6. Selection of O. niloticus to low temperatures; selection intensity ca 20%.

Test No. (No. fish per aquarium)	No. of fish recovering					
Aquarium No.	1	2	3	4	5	
Temp (°C)						
1 (40)	15.0	Beginning of experiment				
	7.0	All fish unconscious				
	8.0	8				
	9.0		8		8	
	10.0					
	11.0					
	12.0		8			
2 (55)	15.0	Beginning of experiment				
	7.0	All fish unconscious				
	6.0					
	9.0	6				
	10.0	5	11	8	12	
	11.0		2	4	4	
	12.0		7			

Figure 3a.

Repeated Cooling Test (March 8, 1988)

First cooling test (February 3, 1988)

Figure 3b

Repeated Cooling Test (March 8, 1988)

First Cooling Test (January 27, 1988)

between these two groups may be due to differences in genotype or size of fish or, more probably, both. The selected (upper 20 percent), and non selected samples were segregated and retained for broodstock for future breeding studies.

Phenotypic response to slow cooling: variation within and among populations

Mean minimum lethal temperatures (MMLT), and mean lethal degree hours (MLDH) were quantified for four normally pigmented species and four red hybrids (Table 7). Rankings of cold tolerance among species for both variables were identical to rankings presented for the short-term studies, i.e., aureus niloticus mosambicus hornorum ($P < 0.05$).

Mean lethal degree hours, an index which incorporates both temperature and time data (summation of degree hours below acclimation temperature), provided better resolution between and within populations than MMLT. For example, the absolute difference in MMLT among species was 4.3°C , a relative difference of 40.2 percent: $((10.7-6.4)/(10.7)) \times (100)$. In contrast, the absolute difference in MLDH was 717^o hours, with a relative difference equal to 70.2 percent. Thus, MLDH can provide better resolution for selection programs and is probably a superior quantitative descriptor of cold tolerance.

Individual body weight within populations was not correlated or was only weakly correlated with MLDH (Table 7). Sex (gender), was not a significant factor affecting MLDH in the four species ($F = 1.51$, $P > 0.22$), or the four red hybrids ($F = 0.85$, $P > 0.35$). The interactions sex x species and sex x hybrid also were not significant factors affecting MLDH ($F = 0.13$, $P > 0.93$) and ($F = 0.41$, $P > 0.74$), respectively.

Table 7. Mean minimum lethal temperatures and mean lethal degree-hours for tilapia fingerlings subjected to a conventional long-term cold tolerance test. Temperature was decreased from acclimation temperature (16°C) to 5°C at a rate of 1°C/day .

Species/hybrid	n	Weight range (g)	Mean minimum lethal temperature ($^{\circ}\text{C}$)*	Mean lethal degree hours (MLDH)	Correlation (r) weight: MLDH
<u>Normally pigmented species</u>					
<u>O. aureus</u>	75	2.6-16.6	6.4 a	1021.0 a	0.25 ($P < 0.03$)
<u>O. niloticus</u>	75	4.9-21.4	7.4 b	788.0 b	-0.05 ($P > 0.60$)
<u>O. mosambicus</u>	75	2.9-34.7	9.5 c	413.0 c	-0.15 ($P < 0.20$)
<u>O. hornorum</u>	75	2.8-28.2	10.7 d	304.0 d	-0.04 ($P > 0.70$)
<u>Red hybrids</u>					
Red hybrid (a) ¹	75	2.2-17.0	6.7 a	945.0 a	-0.26 ($P < 0.03$)
Red hybrid (n) ²	75	2.8-33.8	7.4 a	791.0 b	-0.01 ($P > 0.90$)
Red hybrid (m) ³	75	2.0-48.7	8.9 b	509.0 c	0.12 ($P > 0.35$)
F-2 hybrid ⁴	75	5.2-43.2	7.1 a	853.0 ab	-0.45 ($P > 0.73$)

* Means followed by a different letter are significantly different ($p < 0.05$), according to Student-Newman-Keuls Test.

¹ Red aureus hybrid developed by 5 generations of backcrossing red hybrid male to normally pigmented aureus female.

² Red niloticus hybrid developed by 5 generations of backcrossing red hybrid male to normally pigmented niloticus female.

³ Red mosambicus hybrid with ancestry including mosambicus >> hornorum > aureus > niloticus.

⁴ F-2 red hybrid derived from cross red (aureus_{ab5} x red niloticus_{ab5})²

Due to confounding of the experimental design, it was not possible to statistically compare the hybrids with the species. However, pair-wise comparisons of data for MMLT and MLDH, indicates that hybrids were either equal to or intermediate to their respective parental strains, indicating that overdominance (hybrid vigor), was not a factor influencing the trait cold tolerance.

Incidence of the fungus *Saprolegnia* spp. was acute in *O. hornorum* and *O. mossambicus* at temperatures less than 15°C. In several instances, the fungus covered in excess of 50 percent of their body surface area at time of death. Incidence and severity of fungus infection in *O. aureus*, *O. niloticus*, and their hybrids was comparatively light. Undoubtedly, *Saprolegnia* contributed to deaths, and thus should be considered an important component of cold tolerance and selection.

Phenotypic response to slow cooling: variation among families of O. aureus

Family means for cold tolerance as a function of temperature at lose of consciousness, ranged from 5.7 to 6.8, while cooling degree hours (summation of cooling degree hours below 15° C.) ranged from 805 to 985 (Table 8).

The statistical model used in analysis of cold tolerance data indicated that family means accounted for 30 to 39 percent of the phenotypic variation, while the major reservoir of variation resided among individuals within families. However, the relative merits of individual vs. family selection can only be assessed after heritability estimates are calculated for both methods of selection. The most rapid gains in selection however, may be accomplished by resorting to combined selection, wherein both individual and family selection are practiced simultaneously (Falconer 1982).

Table 8. Cold-tolerance parameters for 20 full-sib families of *O. aureus*. Degree hours represent summation of degree hours below 15°C. Lower critical temperature represents temperature at which fish lose consciousness due to cold narcosis.

Family	Degree house (Base 15)			Lower critical temperature (/C)		
	N	AVG	STD	N	AVG	STD
1	51	934.0	58.3	51	6.1	0.53
2	52	949.3	74.81	52	6.0	0.55
3	18	805.4	287.26	18	6.8	1.65
4	53	909.3	66.08	53	6.2	0.39
5	54	916.3	75.97	54	6.3	0.51
6	52	884.2	80.12	52	6.4	0.49
7	50	822.0	91.40	50	6.6	0.47
9	50	836.7	94.96	50	6.5	0.50
10	50	851.8	73.55	50	6.5	0.41
12	48	863.2	73.61	48	6.5	0.37
13	48	854.5	80.07	48	6.4	0.43
14	46	888.2	88.64	46	6.4	0.54
15	49	900.4	68.42	49	6.3	0.43
16	49	903.5	66.20	49	6.3	0.49
17	48	912.7	74.95	48	6.2	0.53
18	50	893.3	78.50	50	6.3	0.50
19	24	898.3	76.19	24	6.3	0.57
21	50	859.7	75.31	50	6.4	0.41
22	49	822.1	102.62	49	6.6	0.50
23	20	985.0	58.27	20	5.7	0.48

Phenotypic response to slow cooling: survival of O. niloticus (Ghana strain) fingerlings previously selected by the rapid cooling method

The results of the three experiments are presented in Figures 5-7. Slight variation existed among temperature profiles with respect to the lowest temperatures reached (7°C in the first trial, 8°C in the second and third trial respectively), and in the rate of heating. Also, some mortality occurred in the second trial during the acclimation period.

In this latest series of experiments, no real differences in cold tolerance were demonstrated among progeny of select and non-select groups. This may be due to three possible, but not mutually exclusive reasons:

1. Lack of sensitivity of the testing system. In the first and third experiments mortality was close to complete. A difference in cold tolerance between the groups might feasibly have been demonstrated under less severe conditions. Some survival occurred in the second experiment accompanied by slight, though not very convincing, advantage in survival of the progeny of selected fish.

2. In the present generation, selection for survival at low temperatures was carried out by the "fast cooling" method. It is possible that tolerance to fast and slow cooling are two different genetic traits and that selection for one trait does not improve tolerance to the other.

3. The lack of response to selection may be due to low heritability of this trait in the Ghana strain of O. niloticus.

Figure 5. Response to selection for cold tolerance in O. niloticus (Ghana strain). Parents were selected via the rapid cooling technique. Response of progeny tested via slow cooling method.

Figure 6. Response to selection for cold tolerance in *O. niloticus* (Ghana strain). Parents were selected via rapid cooling technique. Response of progeny tested via slow cooling technique.

Figure 7. Response to selection for cold tolerance in *O. niloticus* (Ghana strain) Parents were selected via the rapid cooling technique. Response of progeny tested via slow

Heritability estimates for cold tolerance in tilapias

Table 9 summarizes cold tolerance means, standard deviations, coefficients of variation and selection differentials for three base populations and their respective bidirectionally selected subpopulations. Tables 10 and 11 summarize analogous data for progeny from select and control populations for generation one and two respectively.

Based on a single generation of bidirectional selection for cold tolerance in tilapias, realized heritability estimates were calculated according to the ratio response:selection differential. The ratio can range from -1.0 to 1.0. The estimates and respective standard errors, based on cumulative degree hours, are summarized in Table 12. Estimates for up-selected populations (most cold tolerant) were: *O. aureus*, 0.23; *O. niloticus* -0.048; and F-3 red hybrid, 0.16. Estimates for two down-selected populations were: *O. aureus*, 0.016; and *O. niloticus*, 0.42.

Second generation selection results (Table 11), revealed that up-selection did not lead to further improvements in cold tolerance among any of the selected populations. However, significant responses were evident in down-selected lines of *O. niloticus* and the interspecific hybrid. Lack of response to up-selection may be due to a combination of factors including: sampling error, mistakes in freeze brand identification, and/or a relaxing of the intensity of selection. It should be noted that the selection differential in the first generation was based on the upper and lower 10 percent of a base population consisting of 600 individual. However, selection differential in the second round of selection was based on the upper and lower 50 percent of 200 individuals/population. These individuals consisted of the progeny from selected parents of the base population. Thus the intensity of selection in the second generation was reduced significantly.

Table 9. Cold-tolerance data for three populations of tilapia (and selected subpopulations) subjected to temperature decline of 1°C/day.

Parameter	Mean lower ^{1/}				Cumulative degree hours				Selection differential
	critical temperature (°C)				(Base 15°C)				
	n	x	S.D.	C.V.	n	x	S.D.	C.V.	
<u>Populations</u>									
<u>O. aureus</u>									
Base	594	6.8	0.44	6.5	594	739	72.2	9.8	-
Up-selects (10%)	60	6.5	0.14	2.1	60	844	13.8	1.6	+105
Down-selects (10%)	56	7.6	0.52	6.8	56	593	72.3	12.2	-146
<u>O. niloticus</u>									
Base	584	7.9	0.56	7.0	584	540	69.0	12.8	-
Up-selects (10%)	60	7.4	0.12	1.6	60	644	19.6	3.0	+104
Down-selects (10%)	60	8.8	0.40	4.5	60	415	43.1	10.3	-125
<u>Red Hybrid^{2/}</u>									
Base	603	7.8	0.71	9.1	603	552	97.3	17.6	-
Up-selects (10%)	60	7.0	0.46	6.6	60	698	19.6	2.8	+146
Down-selects (10%)	60	9.0	0.69	7.7	60	366	68.9	18.8	-186

^{1/} Temperature at which fish lose consciousness due to cold narcosis.

^{2/} F-2 hybrid derived from cross (red *O. aureus* ×₅ red *O. niloticus*)₅.

Table 10. Cold-tolerance parameters (means $\pm$ 1 S.D.) for progeny from selected and control populations of *Q. aureus*, *Q. niloticus*, and a red F-3 hybrid (*Q. aureus* $\times$ *Q. niloticus*).

Population	Mean Lower ¹ Critical Temperature (°C)				Cumulative degree hours (Base 15°C)		
	n	$\bar{x}$	S.D.	C.V.	$\bar{x}$	S.D.	C.V.
<i>Q. aureus</i>							
Control	204	6.4	0.49	7.6	853.5	75.22	8.8
Up-select	189	6.2	0.59	9.3	888.7	85.48	9.6
Down-select	208	6.4	0.72	11.3	847.6	114.99	13.6
<i>Q. niloticus</i>							
Control	198	7.1	0.64	9.1	715.1	91.39	12.8
Up-select	209	7.1	0.52	7.2	709.5	74.31	10.5
Down-select	196	7.3	0.60	8.1	662.2	93.22	14.1
<i>F-3 hybrid</i>							
Control	148	7.2	0.56	7.8	699.5	77.99	11.1
Up-select	172	7.0	0.67	9.6	745.1	82.45	11.1
Down-select	-	-	-	-	-	-	-

¹ Temperature at which fish lose consciousness due to cold narcosis.

² Down-selects lost to oxygen depletion in nursery pond.

Table 11. Cold tolerance data¹ (means $\pm$ 1 S.D.) for progeny of three populations of tilapia after two generations of selection². Means within columns followed by different letters are significantly different ($P < 0.05$).

Populations	Mean Lower Critical Temperature (°C)				Cumulative Degree Hours (Base 16°C)			
	n	$\bar{x}$	S.D.	C.V.%	n	$\bar{x}$	S.D.	C.V.%
<i>Q. aureus</i>								
Up-selects	53	6.6a	0.63	9.5	53	1043a	122.7	11.8
Control	44	6.4a	0.63	9.8	44	1089a	118.2	10.9
Down-selects	42	6.4a	0.57	8.9	42	1073a	113.9	10.6
<i>Q. niloticus</i>								
Up-selects	46	7.2a	0.40	5.5	46	920a	74.6	8.1
Control	44	7.2a	0.45	6.2	44	906a	79.0	8.7
Down-selects	50	7.4b	0.61	8.2	50	853b	98.1	11.5
Red Hybrid (F-4) ³								
Up-selects	51	7.2a	0.96	13.4	51	931a	148.0	15.9
Control	50	7.1a	0.57	8.0	50	927a	102.8	11.1
Down-selects ⁴	49	7.3a	0.51	7.0	49	870b	85.3	9.8

¹ Temperature decreased 1°C/day from 16°C to 5°C.

² Intensity of selection in generation one equal to 60/600; generation two 100/200.

³ F-4 hybrid derived from cross (red *Q. aureus* $\times$ red *Q. niloticus*)

⁴ Down selects derived from lower 50% of the previous up-select generation.

Table 12. Response to bidirectional selection for cold tolerance in tilapias. Parameter measured was cumulative degree hours below acclimation temperatures (13-16°C). Standard errors¹ calculated according to Prout (1962).

Population	Generation I				Generation II		
	Selection Differential	Response	Realized Heritability (R/S)	Standard Error	Selection Differential	Response	Realized Heritability (R/S)
<u><i>O. aureus</i></u>							
Up-selects	+105	+35.2	0.33*	0.004	+46	-46.0	-1.00
Down-selects	-146	- 5.9	0.04	0.007	-59	-16.0	0.27
<u><i>O. niloticus</i></u>							
Up-selects	+104	- 5.6	-0.05	0.016	+37	13.0	0.35
Down-selects	-123	-32.9	0.421*	0.004	-72	-53.0	0.74
<u>F3-4 Hybrid</u>							
Up-selects	+146	+45.6	0.311*	0.002	+54	4.0	0.08
Down-selects	-186	-----2	-----	-----	-543	-56.8	1.05

1 Standard error formula only applicable for first generation of selection.

2 100 percent mortality in fingerling production phase.

3 Down-selects in generation II derived from lower 50 percent of the previous generation up-select population.

* Significant response to selection.

These first-ever heritability estimates for cold tolerance in tilapias indicate that at relatively high intensities of individual selection, statistically significant responses can be obtained. However, the magnitude and direction of the response may be species or strain specific. For instance, our results indicate that selection may be useful for improving cold tolerance in *O. aureus* and its hybrids, but not in *O. niloticus*. However, relatively cold-intolerant strains of *O. niloticus* and the interspecific hybrid may be developed via down-selection, but not cold-intolerant strains of *O. aureus*. Cold-intolerant strains of tilapia may be desirable in states or regions where there are restrictions against importation of relatively cold tolerant strains (Kingsley 1987).

Bi-directional selection for cold tolerance in tilapias: correlated responses in growth at suboptimum temperatures

At rearing temperatures of 17.2°C., absolute growth rates (g/individual/month) of selected subpopulations of *O. aureus* and *O. niloticus* were minimal, ranging from -0.04 to 0.29 g/individual/month (Table 13). Although there was little divergence of growth rate among selected subpopulations at this low temperature, it was apparent that growth of *O. aureus*, irrespective of selection criteria, was superior to *O. niloticus*. Similarly, *O. aureus* out performed *O. niloticus* at 19.2 and 21.2°C. This is in contrast to growth performance at higher temperatures (26 to 32°C), where *O. niloticus* generally grows faster than *O. aureus*.

Divergence of growth rates among selected populations within species was weakly manifested at 19.2°C ($P > 0.05$), but readily apparent ($P < 0.05$) at 21.2°C (Table 13). Results indicate that directional selection for cold tolerance in two species of tilapia significantly influenced absolute growth rates at suboptimal temperatures ($P < 0.05$). However, in several instances the results were unexpected. While there was no significant response to two generations of up-selection for the trait cold tolerance in *O. niloticus* (Table 12), there was a noticeable improvement in growth rate of the up-selected population at suboptimal growth temperatures (Table 13). Conversely, two generations of downward selection for cold tolerance resulted in a subpopulation of *O. niloticus*, that was significantly less cold tolerant than its respective control (Table 11). Despite this fact, growth rate at suboptimum temperatures was not significantly influenced by downward selection (Table 13).

Table 13. Growth rate data for selected¹ populations of tilapia cultured at suboptimal temperatures. Each mean based on three replicates of 20 fish each. Growth trials at each temperature regime were conducted for 30 days.

Population	17.2°C			19.2°C			21.2°C		
	Initial Wt. (g)	Final Wt. (g)	Difference ² (g)	Initial Wt. (g)	Final Wt. (g)	Difference (g)	Initial Wt. (g)	Final Wt. (g)	Difference (g)
<i>O. aureus</i>									
Up-select	3.11	3.30	0.19a	3.30	3.65	0.35a	3.65	4.71	1.06a
Control	2.62	2.91	0.29a	2.91	3.17	0.26a	3.17	3.94	0.77b
Down-select	3.04	3.22	0.18a	3.22	3.40	0.18a	3.40	4.15	0.75b
<i>O. niloticus</i>									
Up-select	2.99	2.95	0.04a	2.95	3.12	0.17a	3.12	4.03	0.91a
Control	3.08	3.17	0.09b	3.09	3.21	0.12a	3.23	3.72	0.49b
Down-select	2.99	3.09	0.10b	3.09	3.15	0.06a	3.20	3.77	0.57b

¹ Bidirectional selection for cold tolerance applied for two generations (see text for details).

² Means followed by a different letter are significantly different ($P < 0.05$); vertical comparisons within species only.

Two-dimensional electrophoresis: analysis of liver and blood serum from selected populations of tilapia

The TVA code for the tilapia plasma and liver samples was provided at the conclusion of the electrophoresis experiments. The water temperature (control or treatment), genetic selection group, as well as the TVA code and UAB gel codes for each fish and tissue are combined in Appendix Table 1. Each group was comprised of 5 fish.

Appendix Table 2 summarizes the sex, weight of the fish, liver sample weight and hematocrit data for all of the samples analyzed. Many blood samples were hemolyzed, however, their respective plasma samples were treated as regular samples.

Usual problems were encountered with both first and second dimension separations. A recurring problem with the first dimension was streaking of proteins through the gels. This resulted in horizontal streaking in the second dimension gels. Longer focusing times did not seem to resolve the problem nor did longer centrifugation of the starting sample to remove protein aggregates. Five samples comprising a fish group were always run in parallel (triplicate tube gels) and one or two samples would focus well while other would smear.

Problems with second dimension separations are evident in some gels with multiple vertical smears. Again, some samples separated on the basis of molecular weight ideally while others did not. The built-in molecular weight standards generally indicated the overall value of the gel separation. Some samples focused ideally in the first dimension and streaked in the second dimension while other samples were just the opposite.

The high lipid content of the plasma and liver samples was suspected of causing many of the problems. However, it should be noted that many serum proteins exist as a large number of differently charged molecules and will appear as horizontal smears in the second dimension if not completely resolved in the

first dimension. This is true of IgG, both types of light chains of IgG, fibrinogen, alpha-2 macroglobulin, haptoglobin, etc. The 2-D plasma protein patterns of tilapia compare closely with 2-D patterns of human plasma (Anderson, L. & N.G. Anderson. 1977, Proc. Natl. Acad. Sci. 74:5421-5425).

The non-ionic detergent Triton X-100 was used in initial experiments in the standard protocol. Fuzzy spots, streaking and poor separation suggested high levels of lipoproteins in the samples and that another detergent was perhaps needed. An evaluation of different protein solubilizing/denaturing agents indicated that NP-40 and a saturated urea environment provided the best results for both plasma and liver samples. Although the sample preparation protocols were changed during the project, useful data was present in some of the early gels.

The 2-D separations gave the best and most consistent results with plasma samples in 7 percent gels. Conventional orientation of 2-D gels requires the acidic end of the tube gel to be at the left. It is possible to do this in most cases since the SDS used to chemically denature (reversible) the sample for best IEF separation, electrophoreses away from the proteins and bands at the acidic end near pH 3. It absorbs water and swells the gel slightly and has a faint yellow color in the equilibrating buffer. In some cases it was impossible to tell which end of the gel was acidic and so the 2-D patterns are "backwards". Since the major components of plasma all have molecular weights below about 125 kD, 7 percent gels were chosen for optimal separation. Seven percent gels at 2.6 percent cross linking provides a useful molecular weight separating range of approximately 8-300 kD with an exclusion limit of about 450 kD. By contrast, 12 percent gels at 2.6 percent crosslinking, used for the liver samples, have a useful separating range of 8-100 kD with exclusion of proteins above about 150 kD.

Hemolyzed blood samples provided a useful marker for 2-D gels. Hemoglobin (Hb), with a molecular weight of approximately 16 kD, provides a long streak across the lower part of the gel with a major spot at an estimated isoelectric point (pI) of approximately 5 (Figure 8a). This is lower than human beta and alpha Hb chains with their much more basic pI.

Figures 8a and 8b illustrate some of the problems encountered. "Fingerprints" of *O. niloticus* plasma, show a major vertical streak towards the right side of the gel. This represents material that did not electrofocus in the first dimension. Note also other horizontal and vertical streaks which can be caused for a variety of reasons. The smallest amount of sample was used to control protein-protein aggregation, gel overloading and streaking. Hemoglobin from hemolyzed blood provided a useful internal marker. Molecular weight markers are shown on the right side of the gels.

Results of the cold tolerance study are shown in Figures 9 a-c and 10 a-c with selected photographs of gels of 2-D separation of plasma and liver. Unfortunately, acceptable separations were not obtained for the 15.2 C plasma samples. Figures 9 a-c illustrate fingerprints of the UU, DD and CD groups. Several consistent differences were noted as indicated in three areas on the photographs. The most distinctive differences are indicated by the arrows with the UU group displaying two proteins in the circle to the left and one dominant protein in the top of the circle to the right. The CD group had a major protein (indicated by the arrow) that was not present in the other two groups.

Figures 10 a-c illustrate UU, DD and CD groups adapted to 9.5°C. The UU group still had the set of 2 proteins indicated at the left while all three groups had a new protein at the bottom center. This new protein moved with the dye front which indicates a molecular weight less than 8 kD. The sets of proteins in the circle and box to the right were identical to those present in the 15.2 C groups. However, compared with the intensity of other proteins, one protein in the CD circle was much increased in concentration.

Figures 11a and 11b illustrate differences in fingerprints from fish at 15°C as compared with a surviving group at 5°C. The most notable differences include numerous low molecular weight proteins with a range of isoelectric points from approximately 4- 7.

Figures 12 a-c compare fingerprints among UU, DD and CD groups adapted to 9.5°C. The DD and CD groups are quite different and have present a major protein of approximately 40 kD. Its streak across

the gel probably represents a charge distribution, (possibly due to various degrees of glycosylation). This same protein is present in the liver samples of the UU, DD and CD groups. The fact that this protein is not present in the plasma of the UU group strongly suggests that it is a liver-produced protein. It is possible that this protein is not "leaked" into the plasma of the UU group because they are more "cold tolerant" and/or less stress responsive. It is also possible that the protein is synthesized faster in the DD and CD groups (due to higher sensitivity).

The major new protein appearing in tilapia liver and plasma following adaptation to lower temperature appears to be a new protein and not a breakdown product of existing proteins. There are many other new proteins in the plasma of the 9.5°C DD and CD groups (as well as the 5°C group) but they appear in lower concentrations. In general, they are of lower molecular weight and have pI's from 3 to 7.

Collectively, these illustrations (Figures 9-12), may indicate differential expression of cold tolerance as a response to environment and directional selection. However, sampling error and random drift could also account for these differences and should not be discounted.

Figure 8a
O. niloticus (Egypt)

Figure 8b
O. niloticus x "red" (F-2)

Figure 9a

15.2° C, *O. aureus*
up-select

Figure 9b

15.2° C, *O. aureus*
down-select

Figure 9c

15.2° C, *O. aureus*
control down
(lower 50%) of
control population

Figure 10a
9.5° C, *O. aureus*
up-select

Figure 10b
9.5° C, *O. aureus*
down-select

Figure 10c
9.5° C, *O. aureus*
control-down
(Lower 50% of control)

Figure 11a
15.2° C, *O. aureus*, down-selects

Figure 11b
5° C, *O. aureus*, surviving at end of trial
* Note that gel 11b is reversed, left to right

Figure 12a

9.5° C, *O. aureus*
up-select

Figure 12b

9.5° C, *O. aureus*
down-select

Figure 12c

9.5° C, *O. aureus*
control-down
(Lower 50% of
control population)

DISCUSSION

Phenotypic expression

Phenotypic expression of cold tolerance was strongly influenced by genotypic differences, acclimation history, and the technique by which cold tolerance was measured. By holding acclimation history and technique constant, it was possible to accurately measure phenotypic differences among populations. Similarly, by holding acclimation history and population constant, it was possible to compare phenotypic expression over different test environments.

Rank-order of means among genotypes (species) was identical for the short- and long-term cold tolerance methods indicating no strong GxE interactions. Apparently, time to recovery and MLLT were controlled by common genes, i.e., genes controlling cold tolerance. Thus, it should be possible to use either method to select superior phenotypes for evaluating selection programs. However, from a practical standpoint, each method had shortcomings. The short-term method provides no measurable selection pressure against the fungal infection caused by *Saprolegnia*, spp. This fungus becomes an acute problem under conditions of overwintering at relatively low temperatures. The temperature regime of the long term-method apparently lowers the immunity of tilapia to *Saprolegnia* spp.. Recovery of even slightly infected fish is problematic. Thus, selection for cold tolerance within susceptible populations is complicated by fungus-induced mortality.

Size (range = 2 to 90 g), gender, or color phenotype were not significant factors affecting cold tolerance within populations. Similar results (Behrends and Smitherman 1984), were reported for a red hybrid population derived from *O. aureus* and *O. mossambicus*. Additional research should be conducted to evaluate the relationship between cold tolerance and size of small fry and fingerlings (<1.0g), and individuals larger than 100 g.

Interspecific differences in cold tolerance were well defined with respect to time to recovery, MLLT, and MLDH. Hybrids and products of introgression were either intermediate or equal to parental stains, but in no instance were hybrids superior to the better parent. Lee (1979) also reported no heterosis for cold tolerance among hybrids between *O. aureus*, *O. niloticus*, and *O. hornorum*.

Genetic expression and heritability estimates

Intraspecific differences among geographically distinct strains have been reported for *O. niloticus* (Khater 1985). The most northerly distributed strain (Egypt, 31°N), was significantly more cold tolerant than strains from the Ivory Coast (10°N) or Ghana (6°N). Intraspecific hybridization and backcrossing among the Egypt and Ivory cost stains revealed no significant heterosis (Tave et al. 1990), indicating that cold tolerance in those populations is not controlled by dominance genetic variance. Furthermore, clinal variation among strains within species (Khater 1985), indicates: 1) an intraspecific component of additive genetic variance and 2) that natural selection has operated to extend the range of certain Oreochromid species.

Statistically significant heritability estimates for cold tolerance in tilapias (Table 12) provides experimental evidence that cold tolerance is controlled by additive genes. Therefore, progress in either direction, upward or downward, should be possible at prescribed intensities of selection. The erratic and asymmetrical responses observed within and among species and hybrids (Table 12), during two generations of selection have many potential causes including: random drift, selection differential, inbreeding depression, maternal effects, genetic asymmetry, genes with large effects, scalar asymmetry, and indirect selection (See Falconer 1981, pp. 190-194).

Responses to selection in the first generation were attained at relatively high levels of selection intensity, while responses in the second generation were achieved at much reduced levels of selection intensity. Unfortunately, positive gains made in cold tolerance in up-selected populations of *O. aureus*

and its hybrid during the first generation of selection were lost during the second generation of selection. In contrast, rapid gains in down-selected populations were achieved in *O. niloticus*, irrespective of selection intensity. Such results may indicate that cold tolerance is near a selection limit in these populations. Multiple generations of intense selection however, will be required to confirm this and to determine accurate heritability estimates.

One generalization that Falconer (1981), expounded on, and which may partially explain the asymmetrical responses observed in our studies, is that if the trait under selection is related to fitness, then the following would apply: 1) selection towards increased fitness, ie. cold tolerance, would result in less response than selection towards decreased fitness, 2) traits conferring increased fitness are usually prone to inbreeding depression which is also a cause of asymmetry, and 3) if cold tolerance has been directionally selected via natural selection, then gene frequencies are likely to be above the symmetrical point, and nearer a selection limit, thus resulting in asymmetrical responses.

It should be noted that many of the causes for lack of response and asymmetry of response are exacerbated by small founding populations. In the U.S., certain tilapia populations were founded as early as 1957 with as few as five broodstock (Dr. R.O. Smitherman, personal communication). Also many of the U.S. populations have experienced multiple and severe bottlenecks, further reducing genetic variance. Despite limited genetic variance, we were able to elicit responses to selection.

Our research results revealed several important points that should be assimilated by others undertaking similar studies: 1) cold tolerance is a malleable physiological trait apparently controlled by additive gene action, 2) phenotypic expression is greatly influenced by acclimation history, and 3) within populations, genotypic and phenotypic variances are small, thus precision is required in selective breeding programs.

Because there is considerable clinal variation within certain species, eg. *O. niloticus*, efforts should be made to collect germ-plasm resources from the northern limits of their natural distribution. This could substitute for many generations of costly artificial selection.

Cold tolerance and correlated growth responses

The positive correlated response between cold tolerance and growth at suboptimal temperatures needs to be confirmed by further studies. Although strains up-selected for cold tolerance grew faster than contemporary down-selected populations at suboptimal temperatures, the results were confounded by differences in initial starting weights. Controlling differences in initial weight among test strains either by statistical blocking or mechanical grading is of paramount importance, since small differences in initial weight, an environmental component, can impact growth and thus mask growth differences related to genetic parameters.

Provided that positive correlations exist between cold tolerance and growth at suboptimal temperatures, then in-direct selection programs should be evaluated as a means of selecting for cold tolerance. From a logistical and technical standpoint, this has important implications. Growth at suboptimal temperatures is also a measure of cold tolerance, but is much easier to measure and is less labor intensive than standardized cold tolerance tests. Measurements of growth can also be monitored over longer periods of time, with greater objectivity and precision.

Two-dimensional electrophoresis

This subcontract work was an electrophoretic survey of plasma and liver proteins in cold tolerant-select and control populations in the genus *Oreochromis*. The protein analyzing method, 2-dimensional electrophoresis, is the most sensitive technique known to resolve trace proteins from complex mixtures. The 2-D fingerprints obtained in this study indicate that tilapia respond biochemically to colder temperatures by synthesizing new proteins in the liver. One of these proteins also appears in the plasma in high

concentration and appears to reflect cold stress. Its relationship to developing cold tolerance in tilapia is unclear at this time.

Two major findings were evident from the 2-D analyses. First, tilapia plasma contains many of the same proteins as human plasma with nearly identical isoelectric points and molecular weight. As expected, however, there were many unique tilapia proteins. Perhaps the most surprising difference was that no protein was present (in sufficient concentration to detect) in tilapia which is equivalent by pI and molecular weight to human albumin.

The second finding indicated that a major protein was produced in the liver of tilapia of the DD and CD groups, possibly in response to cold stress. While the protein was present in the liver of the less sensitive fish, it was not present in their plasma. The protein is produced by the liver and exists in multiple molecular forms with virtually the same molecular weight (as is true with a protein with different degrees of glycosylation). Other evidence supporting a glycoprotein is its range down to a pI of 3.

Other differences in protein patterns of the 2-D analyses were noted between the UU, DD and CD groups at 15.2°C. These same differences were also present in all groups adapted to 9.5°C which would suggest that the different patterns reflect genetic drift and/or genetic selection, but not biochemical responses to cold adaptation.

Two dimensional electrophoretic patterns of tilapia adapted to 5°C showed a variety of new low molecular weight proteins not present in controls held at warmer temperature. It is difficult to know the reason for these new isoforms but they appear to be new proteins and not catabolic products of existing proteins. Also, the fact that they tend to have acidic pI's and be of low molecular weight would preclude breakdown of existing proteins. Furthermore, protein denaturing caused by SDS, saturated urea in a strongly reduced environment, and with detergent(s) to solubilize lipids, normally denatures all proteins into peptides and/or subunits.

Finally, it is apparent from the results of this limited study that tilapia respond to cold stress by synthesizing one or more new proteins and that these proteins can be detected readily by 2D electrophoresis in acrylamide gels. Whether the presence or absence of any of these proteins in liver or plasma can be used to predict cold tolerance in tilapia remains to be determined. Further work will be required to determine the identity, structure and function of these novel proteins.

DESCRIPTION OF COOPERATION

During the three years of the cooperative project, communications concerning progress and results were shared in letters or at professional meetings. Formal progress reports were also exchanged, allowing necessary refinements in techniques, and the coordination of complementary studies. Attempts at exchanging stocks, although warranted, was never undertaken. In the U.S., there is no clear understanding (at least on the part of the principal scientist), of the proper protocol for importing stocks for research purposes. However, given the condition of tilapia genetic resources and interest in tilapia culture in the United States and Israel, joint efforts should be attempted at up-grading genetic resources. With respect to cold tolerant forms, a binational team should be selected for a collecting expedition.

The Tennessee Valley Authority closed its Aquaculture Research Unit in 1990, and donated representative stocks of tilapia to Mississippi State University and to a regional consortium composed of three Resource Conservation and Development programs in north Alabama. Stocks were also offered to Auburn University, Alabama, but declined. Specialized equipment will be provided to the RC&D Councils on a no-cost lease basis for research and development purposes.

EVALUATION OF THE RESEARCH ACHIEVEMENTS

A considerable amount of new information was generated during the three years of this research grant. With respect to the initial objectives, U.S. and Israeli counterparts were able to develop and

evaluate both a rapid and slow-cooling cold tolerance test. The fast cooling method allows rapid comparisons among species, strains and families, while the slow cooling method has more application for resolving small differences within populations relative to selection. These standardized tests allow quantifying phenotypic differences in cold tolerance within and among test populations. They can also be used for selecting superior phenotypes for breeding purposes and for testing response to selection in progeny generations. Further research will be required to determine the cross-compatibility of the two methods. However, preliminary data indicates that phenotypic correlations between the two methods are high.

Heritability estimates were calculated for two species and one hybrid population. At rather high levels of selection intensity (generation 1), responses to up-selection were significant ($h^2 = .31$ to $.33$), and population specific. *O. aureus* and the hybrid *O. aureus* x *O. niloticus* both responded to up-selection, while *O. niloticus* (Egypt), only responded to down selection ($h^2 = 0.42$). Similarly, Ghana strain *O. niloticus* did not respond to up-selection.

In the second generation, intensity of selection was relaxed by greater than 50 percent, and up-selection gains from the first generation were lost. However, population continued to respond to down-selection, especially in *O. niloticus*, indicating strong species x selection interaction and strong asymmetry of response. Multiple generations of selection will have to be performed to develop a meaningful realized heritability estimate.

Correlated responses to selection revealed that it may be possible to improve growth at suboptimal temperatures by selecting for cold tolerance. From a practical standpoint, this may provide a marginal increase in the growing season for temperate climates. From a technical standpoint, such a response indicates that indirect selection for cold tolerance may be possible by selecting for growth at suboptimal temperatures. A combination of direct and indirect selection could conceivably result in rapid gains.

Results from two-dimensional electrophoretic studies, while preliminary, hold promise that novel cold tolerance proteins are produced at low temperatures. Verification and identification of such proteins and their respective amino acid sequences will help to lay the groundwork for cloning of cold tolerance genes for future applications in bioengineering.

Development of commercial strains of fast-growing red tilapias with improved cold tolerance remains to be accomplished. Although red "strains" of *O. aureus* have been developed via introgression (Behrends and Smitherman 1984), their growth rate is inferior to *O. niloticus* (Egypt), and their cold tolerance is no better than unselected strains of *O. aureus*. Red "strains" of *O. niloticus* have also been developed via introgression; their growth is very good, however early hatchery mortality and lack of cold tolerance are problematic (Behrends and Smitherman 1990).

Given the apparent lack of additive genetic variance for growth in many of the commercial populations of tilapia, development of inbred lines of fast growing strains of *O. niloticus* should be undertaken, to evaluate crossbreeding and non-additive sources of genetic variation. Tandem development of *O. aureus* improved for cold tolerance via mass and family selection should also be initiated. Terminal crosses between inbred lines and cold tolerant lines would identify commercially valuable hybrid lines.

LIST OF PUBLICATIONS

Behrends, L.L., J.B. Kingsley and M.J. Bulls. 1990. Cold tolerance in maternal mouthbrooding tilapias: phenotypic variation among species and hybrids. *Aquaculture*, 85: 271-280.

Tables and text from this publication were incorporated into the final scientific report.

ACKNOWLEDGMENTS

This research was supported, in part, by a grant from the U.S.-Israel Binational Agricultural Development Fund (BARD US- 1062-86). We are grateful to Ms. Linda Tackett and Mr. Mike Montgomery (TVA Agricultural Institute), and Ms. Nancy Mills (TVA Agricultural Research), for their assistance in typing, editing and formatting of this completion report.

REFERENCES

Barr, A.J., J.H. Goodnight, J.P. Sall, W.H. Blair, D.M. Chilko, K.A. Council and J.T. Helwig. 1979. SAS User's Guide. SAS Institute, Inc., Raleigh, NC. 494 pp.

Becker, W.A., 1984. Manual of Quantitative Genetics. Academic Enterprises, Pullman, Washington. 188 pp.

Behrends, L. L., R. G. Nelson, R. O. Smitherman, and N. M. Stone. 1982. Breeding and culture of the red-gold color phase of tilapia. *Journal World Mariculture Society* 13:210-220.

Behrends, L. L. and R. O. Smitherman. 1983. Use of warm water effluents to induce winter spawning of tilapia in temperate climates. In: Fishelson, L. and Z. Yaron (Editors), International Symposium on Tilapia in Aquaculture. Tel Aviv University, Tel Aviv, Israel, pp. 446-454.

Behrends, L. L. and R. O. Smitherman. 1984. Development of a cold-tolerant population of red tilapia through introgressive hybridization. *Journal World Mariculture Society* (15):172-178.

Behrends, L.L. and R.O. Smitherman. 1990. Genetics and Breeding of red tilapias. Pages 19-26 In R.O. Smitherman and D.A. Tave (Editors), Proceedings Auburn Fisheries and Aquaculture Symposium.

El Gamal, A.A., R.O. Smitherman, and L.L. Behrends. 1988. Viability of red and normal-colored *Oreochromis aureus* and *O. niloticus* hybrids. In The second international symposium on tilapia in aquaculture; 16-20 March 1988, Bangkok, Thailand (R.S.V. Pullin, T. Bhukaswan, K. Tonguthai, and J.L. Maclean, eds.), p. 153-157. Dep. Fisheries, Bangkok, Thailand, and Int. Center for Living Aquatic Resources Manage., Manila, Philippines, ICLARM Conf. Proc. 15.

Falconer, D.S., 1981. Introduction to Quantitative Genetics. Longman Inc., New York, 340 pp.

Gjedrem, T. 1983. Genetic variation in quantitative traits and selective breeding in fish and shellfish. In Genetics in Aquaculture. N.P. Wilkins and E.M. Gosling, (Editors). Elsevier Science Publishers. Amsterdam, Oxford, New York.

Guerrero, R.D., III, 1975. Use of androgens for the production of all male *Tilapia aurea* (Steindachner). *Trans. Am. Fish. Soc.*, 104(2): 342-348.

Khater, A.E. 1985. Identification and comparison of three *Tilapia nilotica* strains for selected aquacultural traits. Auburn University, Alabama. 68 pp. Ph.D. dissertation.

Kingsley, J.B. 1987. Legal constraints to tilapia culture in the United States. *Journal World Aquaculture Society*. Vol. 18, No.3:201-203.

Lee, J.C. 1979. Reproduction and hybridization of three cichlid fishes, *Tilapia aurea*, *T. hornorum*, and *T. nilotica* in aquaria and in plastic pools. Auburn University, Alabama. 84 pp. Ph.D. dissertation.

Myers, J.M. and R. N. Iwamoto. 1986. Evaluation of thermal and chemical marking techniques for tilapia. *The Progressive Fish Culturist*. 48:288-289.

Prout, R., 1962. The error variance of the heritability estimate obtained from selection response. *Biometrics*, 18:404-407.

Shafland, P. L. and J. M. Pestrak. 1982. Lower lethal temperatures for fourteen non-native fishes in Florida. *Environmental Biology of Fishes* 2:149-156.

Tave, D., Jayaprakas, V. and R.O. Smitherman. 1990. Effects of intraspecific hybridization in *Tilapia nilotica* on survival under ambient winter temperature in Alabama. *Journal World Aquaculture Society*. Vol. 21, No. 3:201-204.

Wohlfarth, G. W. and G. Hulata. 1983. Applied Genetics of Tilapias. ICLARM Studies and Review 6. International Center for Living Aquatic Resources Management, Manila. 26 pp.

Appendix 1. Two-dimensional electrophoresis, tissue sample data.

TEMP.	GROUP	TVA CODE	LIVER (L)	PLASMA (P)
17 C		N/1-5 N/6-10	N/5L-9L N/39L-43L	N/15P-19P N/20P-24P
22 C		M/Tave/1-5	MT/59L-63L	MT/25P-28P
22 C	F-2	T x N/1-5	TXN/44L-48L	TXN/29P-32P
19 C	Cont.	A/1-5	A/34L-38L	
19 C	Cont.	M-A/1-5 A/6-10	M-A/103L-107L A/108L-112L	M-A/49P-53P A/54P-58P
15.2C	UU	A/11-15	A/113L-117L	A/64P-68P
15.2C	DD	A/16-20	A/118L-122L	A/69P-73P
15.2C	CD	A/21-25	A/123L-127L	A/74P-78P
9.5 C	UU	A/26-30	A/128L-132L	A/79P-83P
9.5 C	DD	A/31-35	A/133L-137L	A/84P-88P
9.5 C	CD	A/36-40	A/138L-142L	A/89P-93P1
5 C	REM	A/41-45	A/143L-147L	A/93P-97P
16 C	UC	A/46-50	A/148L-152L	A/98P-102P

NOTATIONS:

- A - Oreochromis aureus
- M - " mossambicus
- N - " niloticus
- T - Red mossambica strain provided by DR. Douglas Tave
- T x N - Tave x Q. niloticus
- UU - Upper 50% of up-selected population (10%)
- DD - Lower 50% of down-selected population (10%)
- CD - lower 50% of control
- REM - remainder of fish surviving at 5 C
- UC - upper 50% of control held at 16 C
- Cont. - controls held at ambient temperature

Appendix 2. Two-dimensional electrophoresis, summary of fish and tissue data.

<u>TVA CODE</u>	<u>SEX</u>	<u>FISH WT.</u>	<u>LIVER WT.</u>	<u>PCV/TOTAL BLOOD VOL*.</u>
N/1	M	3.86 g	52 mg	sample too small
N/2	F	2.32	50	7/4 microliters
N/3	F	4.19	48	7/3 "
N/4	F	6.01	105	7/<2 "
N/5	F	3.16	31	h.h.
N/6	F	5.34	112	3/11.2
N/7	F	10.3	118	3.2/14
N/8	F	4.66	38	1/8
N/9	F	4.15	50	<1/8
N/10	F	4.47	80	3.3/13
A/1	M	7.15	633	sample lost
A/2	M	9.14	609	" "
A/3	M	7.89	509	h.h.
A/4	M	5.80	659	h.h.
A/5	M	3.10	380	sample too small
A/6	F	16.2	68	7/13.5
A/7	M	9.3	60	5/12 m.h.
A/8	M	18.6	223	11.5/25
A/9	M	6.7	229	1.5/5
A/10	?	8.0	66	4/9 m.h.
A/11	F	5.7	49	8/15 s.h.
A/12	M	5.1	53	7.5/14.5
A/13	M	4.6	26	3.5/8 s.h.
A/14	M	5.0	32	6/12 w/some RBCs
A/15	M	5.4	47	7.5/13 s.h.
A/16	M	12.2	80	4/9.5 s.h.
A/17	M	5.4	63	4/9.5
A/18	F	13.9	59	6/12.5
A/19	M	6.7	99	6/15.5
A/20	M	4.9	52	6/16
A/21	M	6.6	44	1.5/3
A/22	F	7.4	51	6/12.5
A/23	M	5.6	37	7/15
A/24	M	6.5	27	4.5/10/5
A/25	M	5.0	31	6.5/15
A/26	F	4.6	17	3.5/10 m.h.
A/27	M	4.2	23	3/8 s.h.
A/28	M	4.8	34	4/10 s.h.
A/29	F	4.5	23	5/11
A/30	F	4.2	27	4/9 s.h.
A/31	F	13.1	60	3/18 m.h.
A/32	F	6.0	27	5/15.5 s.h.

A/33	M	5.8	30	8/21
A/34	M	4.2	25	3/7 s.h.
A/35	M	4.2	23	2/10 m.h.
A/36	M	5.3	38	6/11
A/37	F	6.7	27	6/16
A/38	F	8.1	18	7/19
A/39	F	8.5	40	6/14
A/40	?	5.2	18	5/12
A/41	F	4.38	20	3.5/10
A/42	M	4.75	14	3/9
A/43	M	7.11	32	4/11
A/44	?	3.3	23	3/8
A/45	F	6.4	32	4/10 m.h.
A/46	M	4.4	15	3/8
A/47	F	5.5	16	4/9.5
A/48	F	5.6	36	4/10 m.h.
A/49	F	4.5	25	6/13.5
A/50	?	5.6	28	7/14
M/Tave/1	M	44.3	253	6.2/18.5 m.h.
M/Tave/2	F	16.4	104	9/22
M/Tave/3	F	54.6	218	8.2/25
M/Tave/4	F	14.8	31	3.5/8
M/Tave/5	F	34.7	158	no sample
TxN/1	M	23.4	325	2/6.5
TxN/2	M?	21.7	173	6/15
TxN/3	M	14.3	130	3.5/9 m.h.
TxN	?	12.4	88	5/12
TxN	?	15/7	100	7/16 s.h.
M-A/1	F	12.2	97	4.5/12
M-A/2	M	42.6	212	5.1/16.5
M-A/3	M	28.3	219	3/11.5
M-A/4	M	30.0	153	5/19.1
M-A/5	F	10.4	52	2.5/6

A - Q. aureus

N - Q. niloticus

M - Q. mossambicus

T - red Q. mossambicus of Dr. Douglas Tave

TxN - F-2 from cross of Tave red with pure Q. niloticus

M-A - Q. mossambicus (red Arizona strain)

* microliters or millimeters in capillary tube, as noted

PCV - packed cell volume

s.h. - slight hemolysis

m.h. - moderate hemolysis

h.h. - high hemolysis, no hematocrit measurement possible.