



PD/A CRSP SEVENTEENTH ANNUAL TECHNICAL REPORT

METHODS FOR ANDROGENESIS TECHNIQUES APPLICABLE TO TILAPIA

*Eighth Work Plan, Reproductive Control Research 1B (8RCR1B)
Final Report*

William L. Shelton
University of Oklahoma
Norman, Oklahoma, USA

ABSTRACT

Control of reproduction is vital to aquaculture and includes artificial propagation as well as management of unwanted recruitment. Developments in manipulation of the reproductive system provide options to enhance production. Nile tilapia, *Oreochromis niloticus*, spawning was managed by photoperiod and temperature manipulation. A controlled light cycle of 20L:4D and water temperature of $26 \pm 2^\circ\text{C}$ directed spawning to a predictable time frame. A developmental rate (τ_0) relationship was described and applied to chromosome manipulation. Blond Nile tilapia are homozygous recessive for a color mutation that was used as a phenotypic marker in the development of protocol for androgenetic induction. Androgenotes were produced by neutralizing the female genome of normal color Nile tilapia (600 J m^{-2} UV dose), activating the egg with sperm from blond males, and diploidizing with cold shock ($11 \pm 0.5^\circ\text{C}$ for 60 min) applied at various times after incubation at $28 \pm 0.2^\circ\text{C}$. Shock applied at 69 min post-activation produced greater numbers of androgenotes than shocks applied at 59 or 79 min post-activation. Optimization for shock type and associated parameters will be required for production of practical numbers of androgenotes for YY-male breeding programs.

INTRODUCTION

Management of fish reproduction is important to aquaculture; it may involve production of seedstock through artificial propagation or, conversely, through development of limits to recruitment. Enhancement of spawning can be by habitat manipulation or through direct hormonal intervention, while reproductive control also can be direct or based on more complicated modifications (Shelton, 1989; Dunham, 1990). The basic techniques and capacity for control vary between species. Tilapias do not respond well to hormonal induction for spawning control, but unwanted recruitment can be effectively managed by monosexing through direct hormonal treatment. While this direct control is practical, growing concern with the use of steroids in food suggests the desirability of other options. Chromosome manipulation, which includes ploidy alteration or euploidy induction with single-parent genome contribution, can provide alternative control mechanisms. Ploidy manipulation includes the induction of triploids through polar body retention or tetraploidy through interference with first zygotic mitosis. Euploid alterations include gynogenetic (gynogenote) diploidization of the maternal genome through polar body retention (meiogynote) or mitotic interference (mitogynote). Diploidization of the paternal genome (androgenote), or androgenesis, is accomplished by interference with first mitosis for eggs that have been genome neutralized before activation. This report describes the initial development of protocol for the production of androgenetic progeny of Nile tilapia, *Oreochromis niloticus*.

Chromosome manipulation involves one or two basic treatments after obtaining fresh gametes (Thorgaard and Allen, 1986). For androgenesis, the first treatment is the deactivation of the female genome. Ultraviolet irradiation is preferred for simplicity and safety, but also because it dimerizes the DNA rather than fragmenting it. Egg activation with untreated spermatozoa then requires diploidization of the haploid zygote

by some form of shock to interrupt the first mitotic division. Shock is most often physical, e.g., thermal (cold or hot) or pressure. Thermal treatment is usually preferred because of the ease of application and equipment simplicity. In order to prevent chromosome segregation, the shock must be timed to coincide with a cytological event, such as disruption of the spindle fibers during metaphase to prevent karyokinesis, or interference with the cell duplication during cytokinesis. Thus, shock type and intensity, duration, and time of application must be optimally combined into a protocol for maximum yield of diploid progeny. Further, because the rate of development is inversely temperature dependent, either the preshock incubation temperature must be standardized or the shock time must be calibrated to account for the temperature effect. Absolute shock time (minutes post-activation) can be transformed with reference to an index of development rate or mitotic interval (τ_0), also in minutes (Dettlaff and Dettlaff, 1961). Shock time (τ_s) can be related to tau (τ_s/τ_0) to report shock protocol in a dimensionless index which is temperature compensated (Dettlaff, 1986). A tau curve for the Nile tilapia was described during an earlier segment of this study (Shelton, 1999). Finally, these requisite gametic treatments (UV and shock) are near lethal, increasing mortality, and the diploidization of the genome increases homozygosity, thereby reducing fitness and survival.

METHODS AND MATERIALS

Broodstock

The University of Oklahoma stock of *O. niloticus* used in the earlier study segments originated in Ivory Coast and was obtained from Auburn University in 1982. The blond stock was derived from a population in Lake Manzala, Egypt (Scott et al., 1987; McAndrew et al., 1988), and was obtained from Swansea University, UK, in 1987. During various studies the Ivory Coast population became hybridized to an unknown level with the

blond stock. Since this is the phenotypic marker that was to be used in our study, a different stock was obtained for the final series of experiments. Nile tilapia from a Ghana source were obtained from Auburn University in 1998, but the fish in this founder stock were small. They were transferred to ponds for the summer to mature, then overwintered. Fish from this population were first used in experiments beginning in January 1999. Each stock (Ghana, blond, and Ghana × blond) was progeny tested for color phenotype at developmental stages from hatching to swim-up.

Spawning

Spawning of Nile tilapia was managed by photoperiod manipulation, as results of earlier efforts to induce ovulation with gonadotropic injections (Gissis et al., 1988) were inconsistent. Several females (four to six) were stocked with one male in four large aquaria (550 l) or paired in 200-l aquaria. Water was aerated and was circulated at the rate of one turnover per day, and temperature was maintained at $26 \pm 2^\circ\text{C}$. The light cycle was controlled by adjusting a timer to turn on the overhead lighting at 0100 hours; the natural sunset regulated the end of the photoperiod for a total of about 20L:4D. Tilapia spawn about 8 to 10 h after the beginning of the light cycle (Myers and Hershberger, 1991). Ovulation and spawning readiness was judged by courtship behavior, coloration, and papilla erection (Rothbard and Pruginin, 1975; Rothbard, 1979). Females were stripped after initiation of spawning or as indicated by other characteristics that signal ovulation.

Fertilization

For progeny tests of body color and for developmental rate experiments, eggs were collected in a clean container, milt was expressed over the eggs, and water was added directly to initiate activation (Rana, 1988). Fertilized eggs were placed in incubation chambers submerged in a water bath within 2 to 3 min, temperature was regulated closely and 20 to 30 eggs were examined under magnification at intervals of 5 min to record cleavage rate. Developmental rate (τ_0) is defined by the duration of one mitotic cycle during early synchronous cleavage (Dettlaff and Dettlaff, 1961). Tau curves have been used to facilitate chromosome manipulation studies in various fishes (Shelton and Rothbard, 1993; Shelton et al., 1997). The tau curve of Nile tilapia was reported in an earlier progress report (Shelton, 1999); it was described from mean time intervals between the initiation of the first and third mitoses in 5 to 10% of the eggs over a range of temperatures (20 to 30°C). Subsequently, eggs were incubated at $28 \pm 0.2^\circ\text{C}$ in upwelling units of 1-l capacity with sufficient flow to gently tumble the developing embryos. Observations on pigment development were recorded at prehatching, posthatching, and swim-up stages for Ghana, blond, and Ghana × blond. Fish occasionally spawned outside of periods of observation, but fertilized eggs were collected from the brooding females, moved to incubators, and pigmentation development observed.

Chromosome Treatment

Freshly ovulated eggs were stripped from normally pigmented Ghana females (not carrying the blond gene) and allocated into four subsamples. Two to four hundred eggs (about 2 ml) were placed in each of four 10-cm petri dishes with enough 28°C water (10 ml) to provide slight buoyancy. Three subsamples were placed in a UV-crosslinker (FisherBiotech, FB-UVXL-

1000) and exposed to $600 \text{ J m}^{-2} \text{ UV}$; this dosage was determined in an earlier study as sufficient to dimerize the female genome (Shelton, 1999). The UV-treated eggs were activated with freshly collected spermatozoa from a blond male, then maintained at 28°C on a rotating shaker table; the untreated eggs (control) were fertilized with milt from the same male. After 30 min all eggs were transferred from the petri dishes to screened, individual incubator chambers which were submerged in a 28°C water bath. Treated eggs were subsequently plunged into water in a thermal bath at $11 \pm 0.5^\circ\text{C}$ for 60 min; one subsample was cold-shocked at 59 min post-activation, the second at 69 min, and the third at 79 min. The first shock time coincided with the initial mitotic metaphase, the second just prior to cleavage furrow formation (cytokinesis), and the third immediately after cell division. After shock, all eggs were returned to the water bath and then they were transferred, including controls, into individual flow-through incubators. Development was monitored through hatching and swim-up.

RESULTS AND DISCUSSION

Spawning and Progeny Testing

Photoperiod manipulation permitted spawning activity to be forced to mid-day. Females ovulated /spawned between about 9 and 14 h (mean = 11.8 h) after the light-on cycle. Ovulation based on the light cycle was a reasonable means of predicting stripping time. Hormonal induction of ovulation would seem to be a logical extension, but cichlids have responded poorly to gonadotropic therapy (Rana, 1988). During the summer of 1998 while the Ghana broodstock were in ponds, progeny testing of blond stocks continued, then beginning in January 1999, the Ghana stock was incorporated in the spawning efforts. No spawning occurred among the Ghana stock until April, then progeny testing was initiated. Five spawns were sufficient to establish the developmental pattern of pigment for this race. The appearance of melanophores in larval tilapia from normally pigmented broodstock is clearly differentiated from that for blond progeny.

The sequence of melanophore development in four areas is useful for identifying the parental source of progeny; these areas are the yolk, eye, brain surface, and lateral body along the notochord. Progeny of normally pigmented stocks (Ghana) developed melanophores first on the yolk surface—pigment cells could be seen 1 to 2 d prior to hatching. Eye color appeared about 12 h prior to hatch, but was initially diffuse, then developed as a gradual blending of melanin and gold pigmentation. At hatching, scattered chromatin-filled melanophores were present on the dorsal surface of the brain and laterally on the body adjacent to the notochord and near the yolk sac, but subsequently progressed caudad, gradually increased in abundance. In contrast, progeny of blond stocks totally lacked pigmentation in these areas until posthatching, and then coloration was limited to a gold cast in the eyes. Body color did not develop during the larval stages and only non-pigment-bearing melanophores could be seen on the yolk and brain surfaces. These appeared as shadowy cellular outlines that contained no melanin. Finally, hybrids between pigmented females (Ghana) and blond males developed pigmentation in areas and in the sequence described for the Ghana stock. Thus, progeny that have dark coloration in the first several days post-hatching (until the mouth becomes functional) possess genomic contribution from one or both pigmented parents, but not exclusively from blond parent(s); the latter are non-

pigmented throughout larval states. Androgenotes derived from blond male parents can therefore be easily identified from prehatching through swim-up stages.

Chromosome Manipulation

Fertilization rate was variable, with no development in controls (artificial fertilization) of 6 of 17 batches of ova stripped from Ghana females. In the 11 other strip-spawned groups of the control that were artificially fertilized, hatch rate ranged from only 4.0 to 11.3% (mean 10.9%) (Table 1). Motility of spermatozoa was evaluated microscopically and milt was used only if more than 90% of cells were viable. Poor quality ova are most often the primary cause of developmental failures. Initiation of egg resorption is common among tilapias and, although it does not prevent ovulation of the bad eggs, fertilization and hatching are poor (Peters, 1983). Physiological characteristics of tilapia gametes provide advantages in chromosome manipulation. Eggs retain high fertility for 3 to 6 h post-immersion in water (Myers et al., 1995), and sperm remain motile in water for several hours in contrast to most fishes (Yehekel and Avtalion, 1986). Some of the low rate of development may also be attributable to the type of incubation. Hatch rate in the upwelling type of incubator is not as good as in a down-flow system (Rana, 1986); our incubators incorporate this preferred down-flow design.

Hatch rates for UV-treated and cold-shocked ova were significantly lower than those of controls, i.e., never more than a fraction of a percent. Relative to the controls, hatch rate ranged from about 3% of the control for the shock that started

at 59 min after activation to about 22% of the control for the shock that started at 69 min (Table 1). Increased mortality resulting from the UV dose is expected due to cell damage. Successful diploidization varied depending on the proximity of shock to the optimal induction "window." Optimization was not the primary objective during the Eighth Work Plan and only three shock times were tested. The late shock protocol for tetraploid induction that was successfully developed by Don and Avtalion (1988a, 1988b) and Don (1989) was used as the basis for induction of androgenesis. The cold shock has a wider time of application (60 min) compared to heat or pressure shock (2 to 5 min) and intuitively should provide a greater likelihood of some successful diploidization. Further, the shock application time used by the Israeli scientists is based on cytological correlation that relates well to time factors as described by tau adjustments (Saat, 1993; Shirak, 1996; Shirak et al., 1998). This is in sharp contrast to the empirical approach of Hussain et al. (1993), Mair (1993), and Myers et al. (1995), where shock time application had little relationship to cytological events.

Hatch rates of control groups in 11 trials indicated reasonably good quality ova (Table 1). Induction of androgenetic progeny (UV-treated Ghana ova, activated with sperm from blond males and diploidized by cold shock) was tested by cold-shocking at three post-activation times (59, 69, and 79 min at 28°C) which correspond to 0.75, 0.87, and 1.0 T (T = time of first mitosis) or 1.8, 2.1, and 2.5 τ_0 , respectively. The former (0.75 T) is the approximate time for first mitotic metaphase (Saat, 1993; Rubinshtein et al., 1997), while the second (0.87 T) is just prior to first mitotic cytokinesis and the optimal induc-

Table 1. Induction of androgenotes of Nile tilapia (Ghana females x blond males) using cold shock on eggs artificially fertilized (11 ± 0.5°C for 60 min to eggs UV-treated with 600 J m⁻²).

| Trial | Eggs (#) | Control (#) | Treatment (#) | | | Androgenote (#) |
|-------|-----------|-------------|-----------------------------|-------------------------|-------------------------|---------------------|
| | | | Hatch (Survival to Swim-up) | | | |
| | | | 59 min | 69 min | 79 min | Survival to Swim-up |
| 1 | 510 | 25 (12) | 1 (0) | 2 (1) | 0 | 1 |
| 2 | Inviabile | | | | | |
| 3 | 400 | 10 (3) | 0 | 1 (0) | 0 | 0 |
| 4 | 650 | 35 (10) | 1 (0) | 4 (1) | 2 (0) | 1 |
| 5 | Spawn | | | | | |
| 6 | Inviabile | | | | | |
| 7 | Inviabile | | | | | |
| 8 | Inviabile | | | | | |
| 9 | Spawn | | | | | |
| 10 | Inviabile | | | | | |
| 11 | Inviabile | | | | | |
| 12 | 800 | 55 (40) | 2 (0) | 10 (4) | 4 (1) | 5 |
| 13 | Spawn | | | | | |
| 14 | 750 | 85 (62) | 4 (1) | 21 (11) | 10 (3) | 15 |
| 15 | 625 | 42 (21) | 1 (0) | 13 (5) | 7 (2) | 7 |
| 16 | Spawn | | | | | |
| 17 | Spawn | | | | | |
| 18 | 420 | 20 (11) | 0 | 5 (2) | 4 (1) | 3 |
| 19 | 850 | 75 (43) | 2 (0) | 21 (10) | 7 (2) | 12 |
| 20 | 710 | 63 (24) | 4 (0) | 16 (5) | 2 (0) | 5 |
| 21 | 410 | 33 (21) | 1 (0) | 8 (2) | 2 (1) | 3 |
| 22 | 530 | 25 (10) | 0 | 2 (0) | 0 | 0 |
| Sum | 6,655 | 257 = 3.9% | 16 = 3.4% of control | 103 = 22% of control | 38 = 8.1% of control | |

tion time for late cold-shock in tilapia as determined by Shirak et al. (1998). The highest hatch rate of androgenotes (non-pigmented larvae) was at the 69-min post-activation shock time; the other two shock times had lower yields. Induction success for androgenotes to hatching does not ensure survival to later stages. From a total of 157 androgenotes that hatched, only 57 survived to swim-up or yolk absorption (Table 1). Additional mortality during the juvenile stages further reduces the number that will be available for progeny testing to verify the production of YY males. As of this report, only five of these androgenotes have survived to the end of July.

Optimization of shock protocol is affected by the asynchrony in cell division; at 28°C, the time from initiation of first cleavage to end of the last encompasses about 20 min for the Nile tilapia (Shirak et al., 1998). Thus, the shock application and duration will affect only a small percentage of the cells at an optimal induction time. Further, even adjusting for the incubation temperature effect, optimization of treatment time for shock is complicated by apparent differences in effectiveness of various types of shock. Palti et al. (1997) clearly demonstrated a difference in optimal shock time for gynogenetic induction with heat and pressure shock for rainbow trout, *Oncorhynchus mykiss*. Similarly, Hussain (1995) demonstrated differential optima between cold, heat, and pressure shock in Nile tilapia. Shirak et al. (1998) suggest that cold shock interferes with cell division, while the mechanism for heat shock has been considered to be disruption of the spindle fibers during karyokinesis (Mair, 1993).

The most effective type of shock must be determined and must be optimized based on the induction mechanism. Thermal shocks will be used for practical reasons and because effectiveness is as good as or better than pressure shock. Don and Avtalion (1988a) used cold shock for tetraploid induction based on comparative effectiveness for triploid induction (Don and Avtalion, 1988b). However, the timing for shock reported by Myers et al. (1995) at 27 min post-activation (28°C) markedly contradicts the optimal shock time of 92 min post-activation (26°C) reported by Don and Avtalion (1988a) and Don (1989), even when adjusted for the different incubation temperatures. Therefore, these reported induction times and shock types will be examined for optimization in the context of developmental rates (τ_0).

ANTICIPATED BENEFITS

Control of unwanted reproduction in tilapia culture has been one of the primary considerations affecting successful food production. Various approaches have been developed and utilized. Most recently, efforts to develop systems that will allow breeding programs to produce monosex cultures have been sought. Androgenesis offers a mechanism that in theory can directly produce YY-males that can be used as broodstock to breed all-male progeny. Demonstration of the induction technology, with its multiple pitfalls, is the first step. Optimization of treatments must be the next achievement so as to increase the numbers of viable androgenotes for practical survival expectations to test the theoretical basis for sex determination and demonstrate that breeding for a monosex can be accomplished.

LITERATURE CITED

- Dettlaff, T.A., 1986. The rate of development in poikilothermic animals calculated in astronomical and relative time units. *J. Therm. Biol.*, 11:1-7.
- Dettlaff, T.A. and A.A. Dettlaff, 1961. On relative dimensionless characteristics of the development duration in embryology. *Arch. Biol.*, 72:1-16.
- Don, J., 1989. Study of ploidy and artificial induction of gynogenesis in tilapias. Ph.D. dissertation, Bar-Ilan University, Tel-Aviv, Israel (in Hebrew).
- Don, J. and R.R. Avtalion, 1988a. Production of viable tetraploid tilapia using the cold shock technique. *Bamidgeh*, 40:17-21.
- Don, J. and R.R. Avtalion, 1988b. Comparative study on the induction of triploidy in tilapia using cold- and heat-shock technique. *J. Fish Biol.*, 32:665-672.
- Dunham, R.A., 1990. Production of monosex or sterile fish in aquaculture. *CRC Rev. Aquat. Sci.*, 2:1-17.
- Gissis, A., B. Levavi-Sivan, H. Rubin-Kedem, M. Ofir, and Z. Yaron, 1988. The effect of gonadotropin releasing hormone superactive analog and dopamine antagonists on gonadotropin level and ovulation in tilapia hybrids. *Bamidgeh*, 43:123-126.
- Hussain, M.G., 1995. Suppression of meiotic and mitotic cell division in Nile tilapia, *Oreochromis niloticus* L. and induction of triploids and two types of gynogenetic diploids. *Asian Fish. Sci.*, 8:133-142.
- Hussain, M.G., D.J. Penman, B.J. McAndrew, and R. Johnstone, 1993. Suppression of the first cleavage in the Nile tilapia, *Oreochromis niloticus* L.—A comparison of the relative effectiveness of pressure and heat shock. *Aquaculture*, 111:263-270.
- Mair, G.C., 1993. Chromosome set manipulation in tilapia—techniques, problems and prospects. *Aquaculture*, 111:227-244.
- McAndrew, B.J., F.R. Roubal, R.J. Roberts, A.M. Bullock, and M. McEwen, 1988. The genetics and histology of red, blond and associated colour variants in *Oreochromis niloticus*. *Genetica*, 76:127-137.
- Myers, J.M. and W.K. Hershberger, 1991. Artificial spawning of tilapia eggs. *J. World Aquacult. Soc.*, 22:77-82.
- Myers, J.M., D.J. Penman, Y. Basavaraju, S.F. Powell, P. Baoprasertkul, K.J. Rana, N. Bromage, and B.J. McAndrew, 1995. Induction of diploid androgenetic and mitotic gynogenetic Nile tilapia (*Oreochromis niloticus* L.). *Theor. Appl. Genet.*, 90:205-210.
- Palti, Y., J.J. Li, and G.H. Thorgaard, 1997. Improved efficiency of heat and pressure shocks for producing gynogenetic rainbow trout. *Prog. Fish-Cult.* 59:1-13.
- Peters, H.M., 1983. Fecundity, egg weight and oocyte development in tilapias (Cichlidae, Teleostei). *ICLARM Transl. No. 2* (D. Pauly), Manila, Philippines. (Original Publication: 1963, *Int. Rev. Ges. Hydrobiol.*, 48:547-576).
- Rana, K.J., 1986. An evaluation of two types of containers for the artificial incubation of *Oreochromis* eggs. *Aquacult. Fish. Manage.*, 17:139-145.
- Rana, K.J., 1988. Reproductive biology and the hatchery rearing of tilapia eggs and fry. In: J.F. Muir and R.J. Roberts (Editors), *Recent Advances in Aquaculture*, Volume 3. Croom Helm, London, pp. 343-406.
- Rothbard, S., 1979. Observation on the reproductive behavior of *Tilapia zillii* and several *Sarotherodon* spp. under aquarium conditions. *Bamidgeh*, 31:35-43.
- Rothbard, S. and Y. Pruginin, 1975. Induced spawning and artificial incubation of tilapia. *Aquaculture*, 5:315-321.
- Rubinshtein, I., S. Rothbard, and W.L. Shelton, 1997. The relationship between the embryological age, cytokinesis-1 and the timing of ploidy manipulations in fish. *Isr. J. Aquacult. / Bamidgeh*, 49:99-110.
- Saat, T. 1993. The morphology and chronology of oocyte final maturation and fertilization in fish. In: B.T. Walther and J.J. Fyhn (Editors), *Physiological and Biochemical Aspects of Fish Development*. University of Bergen, Norway, pp. 71-85.
- Scott, A.G., G.C. Mair, D.O.F. Skibinski, and J.A. Beardmore. 1987. "Blond": A useful new genetic marker in the tilapia, *Oreochromis niloticus* (L.). *Aquacult. Fish. Manage.*, 18:159-165.
- Shelton, W.L., 1989. Management of finfish reproduction for aquaculture. *CRC Rev. Aquat. Sci.*, 1:497-535.
- Shelton, W.L., 1999. Nile tilapia gamete management for chromosome manipulation. In: K. McElwee, D. Burke, M. Niles, and H. Egna (Editors), *Sixteenth Annual Technical Report. Pond Dynamics /*

- Aquaculture CRSP, Oregon State University, Corvallis, Oregon, pp. 69–72.
- Shelton, W.L. and S. Rothbard, 1993. Determination of the developmental duration (τ_0) for ploidy manipulation in carps. *Isr. J. Aquacult./Bamidgeh*, 45:73–81.
- Shelton, W.L., S.D. Mims, J.A. Clark, A.E. Hiott, and C. Wang, 1997. A temperature-dependent index of mitotic interval (τ_0) for chromosome manipulation in paddlefish and shovelnose sturgeon. *Prog. Fish-Cult.*, 59:229–234.
- Shirak, A., 1996. Chromosome set manipulations in tilapias: A model of lethal gene inheritance. M.S. thesis, Bar-Ilan University, Tel-Aviv, Israel (in Hebrew).
- Shirak, A., J. Vartin, J. Don, and R.R. Avtalion, 1998. Production of viable mitogynogenetic *Oreochromis aureus* using the cold shock and its optimization through definition of cleavage time. *Isr. J. Aquacult./Bamidgeh*, 50:140–150.
- Tave, D., M. Rezk, and R.O. Smitherman, 1989. Genetics of body color in *Tilapia mossambica*. *J. World Aquacult. Soc.*, 20:214–222.
- Thorgaard, G.H. and S.K. Allen, Jr., 1986. Chromosome manipulation and markers in fishery management. In: N. Ryman and F. Utter (Editors), *Population Genetics and Fishery Management*. Washington Sea Grant Program, University of Washington Press, Seattle, pp. 319–331.
- Yeheskel, O. and R.R. Avtalion, 1986. Artificial fertilization of tilapia eggs, a preliminary study. In: Y. Zohar and B. Breton (Editors), *Reproduction in Fish—Basic and Applied Aspects in Endocrinology and Genetics*. Les Colloques de l'INRA, No. 44, Paris, pp. 169–175.

