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Regulated Sex Control in Commercially Important Fishes - A Physiological Perspective

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Key words: sex control, physiology, ploidy manipulations

Abstract

The management of fish reproduction has resulted in major advancements in the commercial culture of fishes. Artificial propagation has stabilized seed-stock production, and reproductive manipulations in induced sex reversal and ploidy manipulations have provided mechanisms to improve yield. The design and effectiveness of these manipulations are regulated by various physiological factors. The effectiveness of protocols for the induction of gynogenesis, triploidy, and tetraploidy is improved through knowledge of physiological effects on important parameters. Application of the developmental rate based on the mitotic interval (τ_o) incorporates a standardization relative to temperature. Timing of shock with reference to the species-specific τ_o relationship is effective in clarification and optimization of treatments. Such standardization is important to any late (endomitotic - Em) shock induction, and in polar-body (Pb) induction for many species. Hormonally induced sex reversal also must be applied relative to an efficacious treatment protocol, developed relative to a window of gonadal lability during the genetically directed chronology and physiologically influenced differentiation. Size and/or age are important modifying parameters that can be affected by various growth-altering environmental factors such as temperature and density-dependent effects. The consideration of influences that affect physiological rates relative to reproductive manipulations provides a more in-depth understanding of protocol effectiveness.

Introduction

Developments in the management of fish reproduction have had major impacts on the growth of aquaculture. The capability to spawn fishes under controlled conditions ensures an adequate supply of young for growout, whether for food or stocking (Zohar, 1989; Yaron and Zohar, 1993). Artificial propagation techniques have facilitated worldwide introduction of important species by removing the geographic constraint of culturing only within their native range (Shelton and Rothbard, 2006). The application of this technology is epitomized by the Chinese carps (Opuszynski and Shireman, 1995), but, even for species such as common carp that will reproduce under most culture conditions, induced ovulation has permitted more efficient management and thereby provided greatly enhanced capacity to perform manipulations and conduct breeding programs (Hulata,

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1995; Rothbard and Yaron, 1995; Gomelsky, 2003).

Manipulations of the component systems in reproductive biology have enhanced yield through the capability to control unwanted spawning, as well as through stock improvements. Control of unwanted reproduction has been instrumental in efficient culture of several species, e.g. tilapias; however, it has been used as a component of introduction protocol in relatively few cases. Direct application of reproductive controls to aquaculture involves management of recruitment and the concomitant growth enhancement (Wohlfarth and Hulata, 1989; Pandian and Koteeswaran, 1998; Hulata, 2001), as well as for the utilization of non-native fishes in an ecologically responsible manner where no reproduction is wanted (Shelton, 1989, 2000). Thus, management of fish reproduction can be considered from these two perspectives: the production of seedstock under controlled conditions, and/or the restriction of unwanted reproductive. Both are valuable for aquaculture, either as a tool in culture or as a potential security measure to avoid naturalization.

This review will focus on mechanisms to change functional phenotype and the physiological processes that affect their induction efficiency (Devlin and Nagahama, 2002). The main theme of the discussion will emphasize how rates of physiological processes should be factored into protocols. For example the temperature effect on the developmental rate relative to timing of ploidy manipulations, and the effect of growth rate on sex reversal treatment. The chronology of ontogenetic processes such as gonadal differentiation and sexual maturity are affected by environmental factors that alter rates of development, consequently any induced manipulations must take appropriate trajectories into consideration (Shelton, 1989).

Time-relationship chronologies are genetically determined and species specific, where developmental milestones occur relatively early in some fishes. Gonadal differentiation is a relatively early post-embryonic process, while initial sexual maturation develops somewhat later (Fig. 1). For example, the gonads of tilapia differentiate at a small size within a few weeks and sexual maturation occurs within a few months, while the gonads of sturgeons and paddlefish differentiate only after a year or more of age and sexual maturity is not reached until fish are large and several years old. These relationships are generally affected by genetic adaptation of each species to environmental temperature (e.g., cold vs. warm water), but they are influenced by environmental factors. Species differences are seen early in the developmental period (Fig. 2). Therefore, specific timing of critical developmental events must be considered within a reproductive management program in order to apply effective treatments for ploidy manipulations, such as the time of 2nd polar body formation, or karyo/cytokineses of first mitosis relative to shock application (Fig. 3). Likewise, the effective induction of phenotypic sex reversal must correlate with the labile period during gonadal differentiation.

Ploidy Manipulations

Chromosome manipulations, including gynogenesis, androgenesis, and polyploidy induction provide numerous options for genetic selection, sterility, and sex control (Thorgaard, 1986; Pandian and Koteeswaran, 1998). Chromosomal manipulations can result in alterations that affect the functionality of phenotypic sex, and the functional sex can be changed by exogenous factors during the developmentally plastic period. The resulting mismatch of genotypic and phenotypic sex (e.g., neomales) can be applied to breeding programs (Shelton, 1986; Trombka and Avtalion, 1993; Pandian, 1999). Devlin and Nagahama (2002) have provided an excellent review of sex determination systems in fishes and how these are transcribed into functional phenotypic sex during gonadal differentiation.

Gynogenesis is defined as activation of gametes without paternal genetic contribution, but where diploidy is induced through retention of the 2nd meiotic polar body or by interference with the initial zygotic mitosis (Thorgaard, 1983). Elimination of the male genome is accomplished by DNA-modification (dimerization or fragmentation) with UV irradiation, X-ray or ionizing radiation treatment

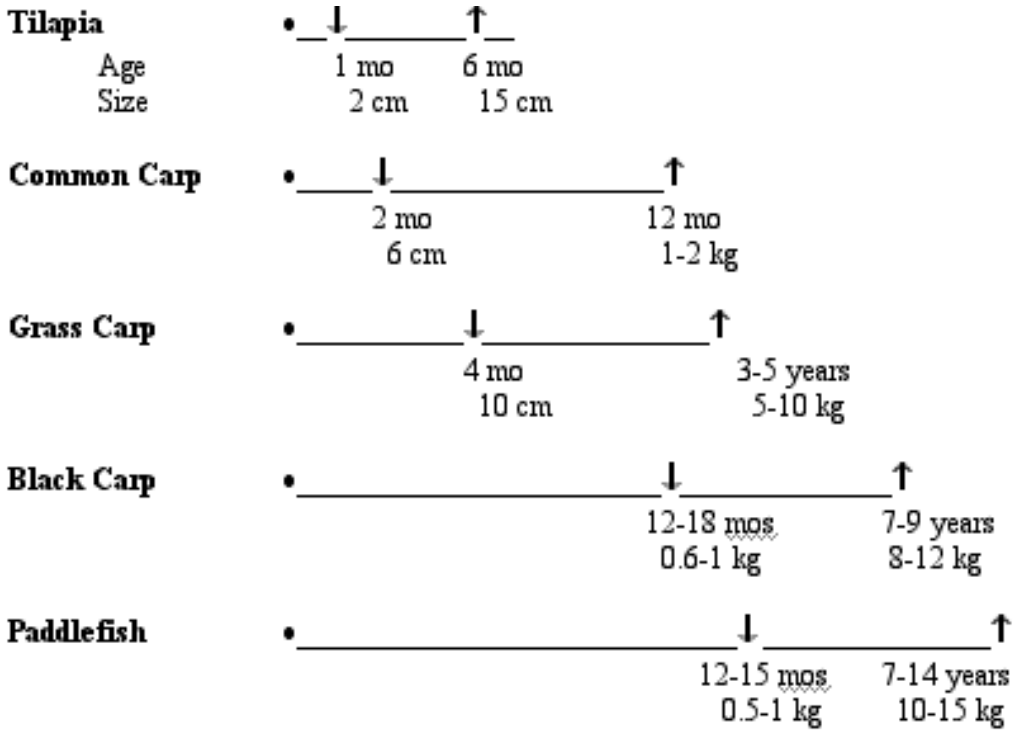

Fig. 1. Chronology of the development of the female reproductive system in representative teleosts. ● = hatching, ⬇ = gonad differentiation, ⬆ sexual maturity.

(Chourrout, 1987). During optimization of the induction protocol, normal progeny can be differentiated from gynogenotes by using an animal model that offers a homozygous-recessive such as a color mutation or scalation. Spontaneous gynogenesis occurs, but diploidization frequency can be increased through various physical shocks; diploid gynogenotes can be induced by early shock (2nd meiotic polar body - Pb), or late shock (endomitotic; Thorgaard, 1996). Gynogenotes are fertile if diploid, and female if sex determination is of the XX-type (Fig. 3). In contrast, polar body retention in normally inseminated gametes (3N) results in sterility; both 3N-sexes are functionally sterile. Fertile tetraploid (4N) males and females can be induced by interference with first mitotic karyokinesis of a diploid zygote.

Artificial gynogenesis and polyploidization have been induced in tilapias (Don and Avtalion, 1988ab; Hussain et al., 1991, 1993, 1996, 1998; Mair, 1993; Saat, 1993; Hussain, 1995), in common carp (Cherfas et al., 1990; Komen et al., 1991; Rothbard, 1991; Horvath and Orban, 1995), and in the Chinese carps with no shock (Stanley, 1976; Shelton, 1986), heat-shock (Cassani and Caton, 1985; Mirza and Shelton, 1988; Rothbard et al., 1997; Rothbard et al., 2000), cold or pressure-shock (Cassani and Caton, 1986; Cassani et al., 1990; Rothbard et al., 2000).

Induction effectiveness for gynogenesis, androgenesis, and polyploidy depends on optimized manipulation of a physical shock characteristic such as type (cold, heat, or pressure), magnitude, or time-related variable (duration and time of application after gamete

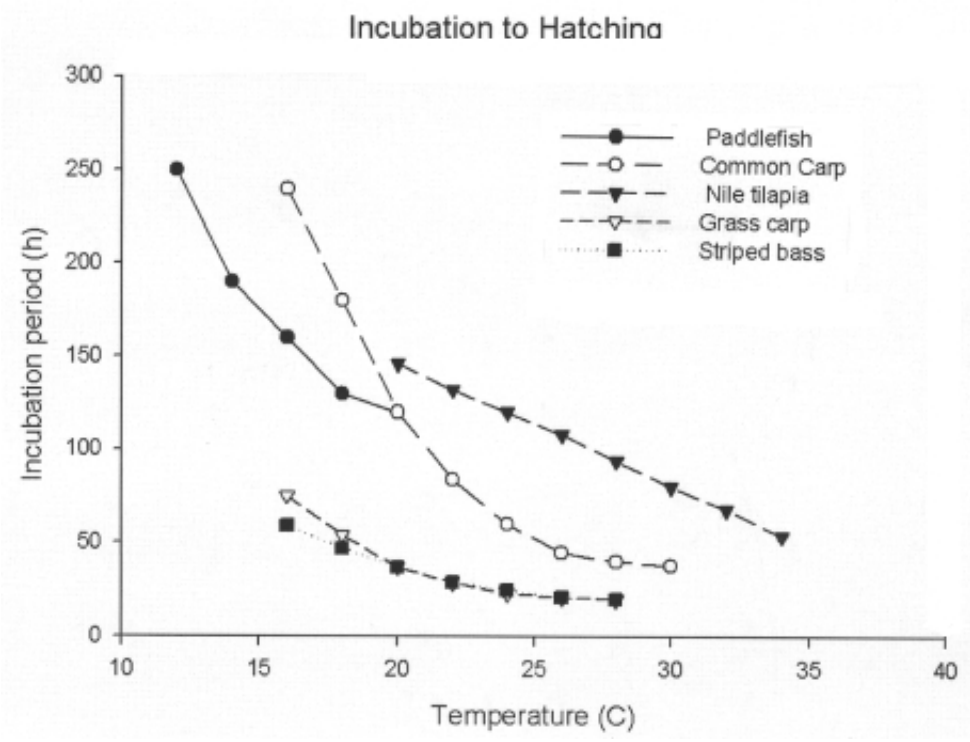

Fig. 2. Incubation period for four warmwater fishes from fertilization to hatching at various temperatures. Data from Setzler et al. (1980), Rana (1988), Rothbard and Yaron (1995), and Mims and Shelton (2005).

activation). Another variable that affects induction efficiency is the inherent asynchrony of cytological events (Shirak et al., 1998). In addition, a related factor is the disparity among populations (Hershberger and Hostuttler, 2005). Pre-shock incubation temperature is important because it affects metabolic and developmental rate, and thus the optimal induction window relative to Pb formation, or nuclear events during first mitosis. Further, there is evidence that the type of shock may affect different events, thereby being optimally effective at different absolute times (Chourrout, 1986, 1987; Palti et al., 1997). The use of mitotic interval (τ_o - min) provides a convenient index to characterize the temperature-related developmental rate (Dettlaff, 1986) and permits standardization of induction protocols for timing the application of shock (τ_s - min).

Representative τ_o-curves and comparable data for time to first mitosis (*T*) in select species are illustrated in Fig. 4. Zygotic cleavage in coldwater fishes is considerably different from in warmwater species, even at comparable temperatures. In rainbow trout, τ_o = 150 min at 14°C compared to 80 min for paddlefish. For Nile tilapia, τ_o = 80 min at 20°C but is only about 12 min for black carp. Thus, for protocol comparability, pre-shock incubation temperature must be reported, or shock initiation referenced to τ_o. Second polar body formation occurs in most warmwater fishes within a few minutes after egg activation, while for others such as paddlefish or sturgeon, Pb-shock (τ_s) must be initiated considerably later (Fig. 5). The utility of the dimensionless unit (τ_s/τ_o) to compensate for different pre-shock incubation temperatures is illustrated by Mims

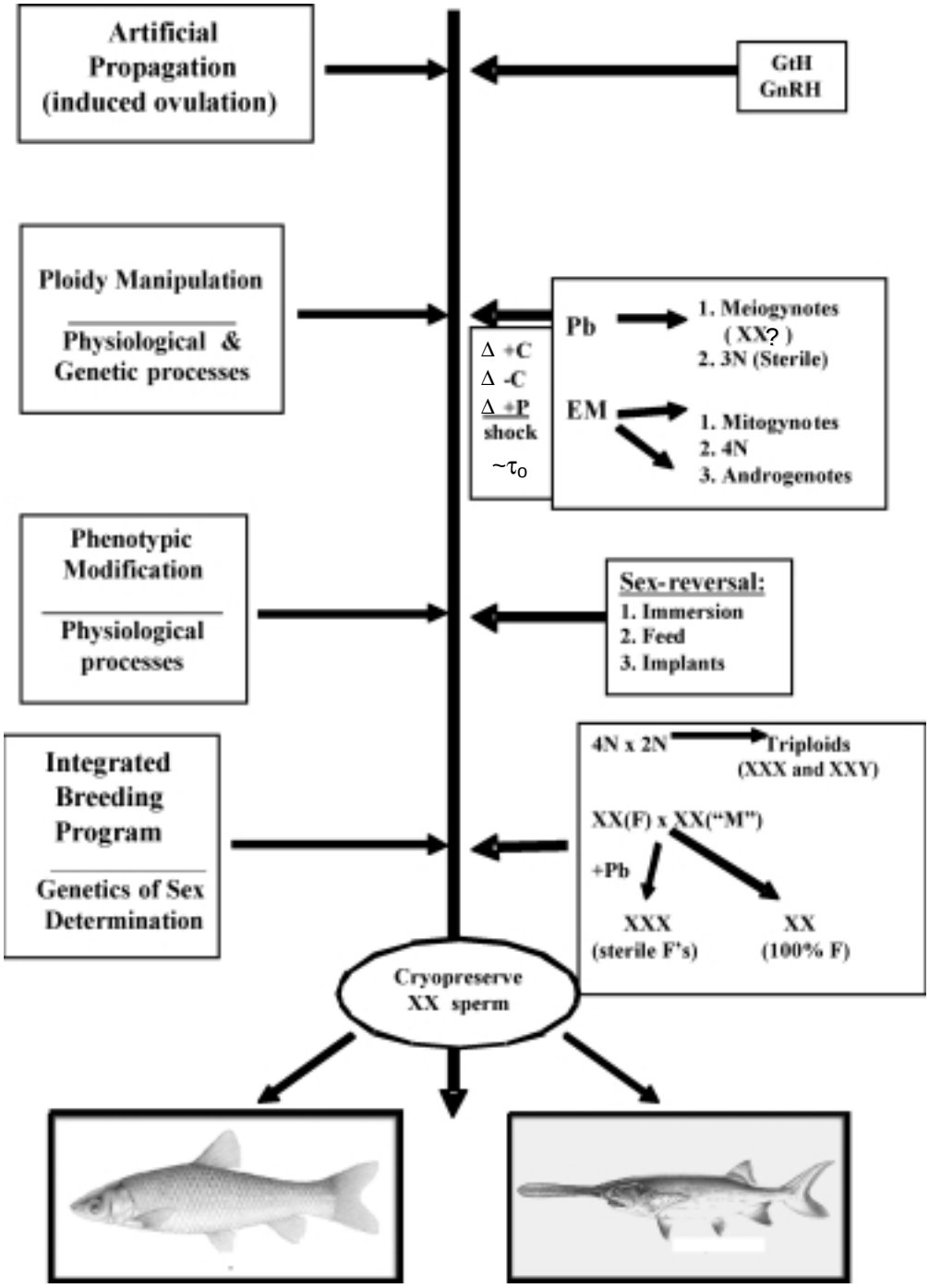

Fig. 3. Management components in a fish reproduction program.

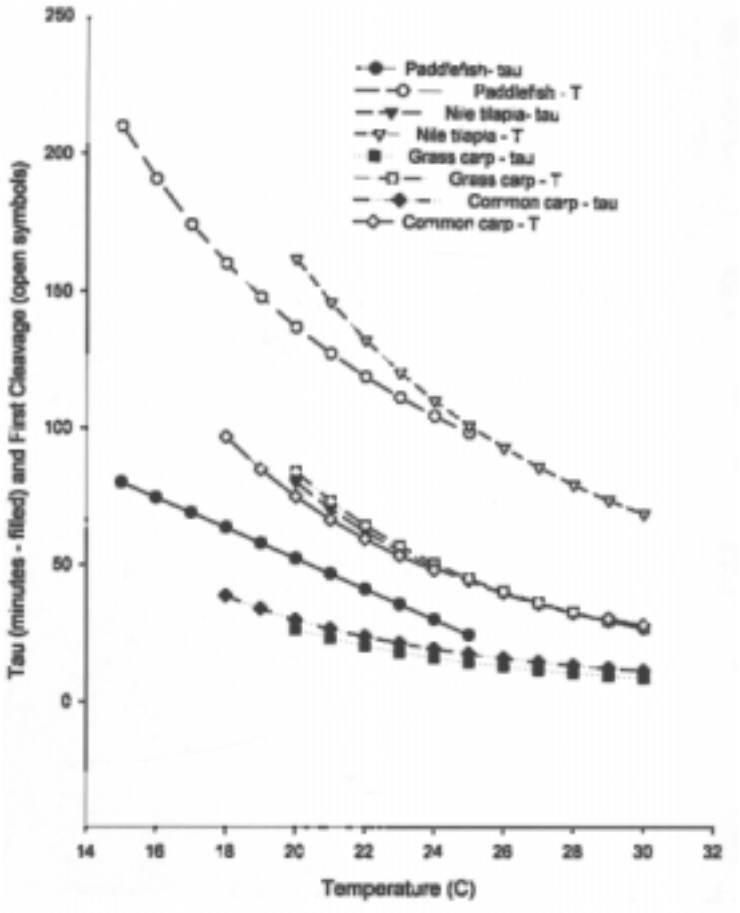

Fig. 4. Mitotic interval (τ_o) and time to first mitosis (*T*) for four species of warmwater fishes. Data from Ignatieva (1991), Shelton and Rothbard (1993), Shelton et al. (1997), and Shelton and Popma (2006).

et al. (2005). Polar-body shock for paddlefish and shovelnose sturgeon were found to be optimal between 0.23-0.28τ_o and 0.28-0.32τ_o, respectively. Van Eenennaam et al. (1996) produced gynogenetic white sturgeon by heat shocking at 0.21-0.26τ_o. Effective early shock for most other warmwater species usually is less than 0.1-0.2τ_o.

Pre-shock incubation compensation is even more critical for late shock manipulations (endomitotic gynogenesis or tetraploidy), regardless of species. Time from insemination to first mitosis (*T*) and mitotic interval during synchronous cleavage (τ_o) and mitotic interval for four warmwater species is illustrated in Fig. 4. Time to *T* is 2-3 times greater than τ_o for warmwater fishes (Shelton and Rothbard, 1993). Saat (1993) indicates that the first mitotic metaphase in teleosts is about 1.5τ_o, whereas the first furrow formation (*T*) during the initial mitotic cytokinesis is about 2.5τ_o. He suggests that the most effective heat-shock application time for carp is during anaphase, or 1.7τ_o, which is approximately 0.68*T*. Gomelsky (2003) summarizes several ploidy studies for common carp and concluded that

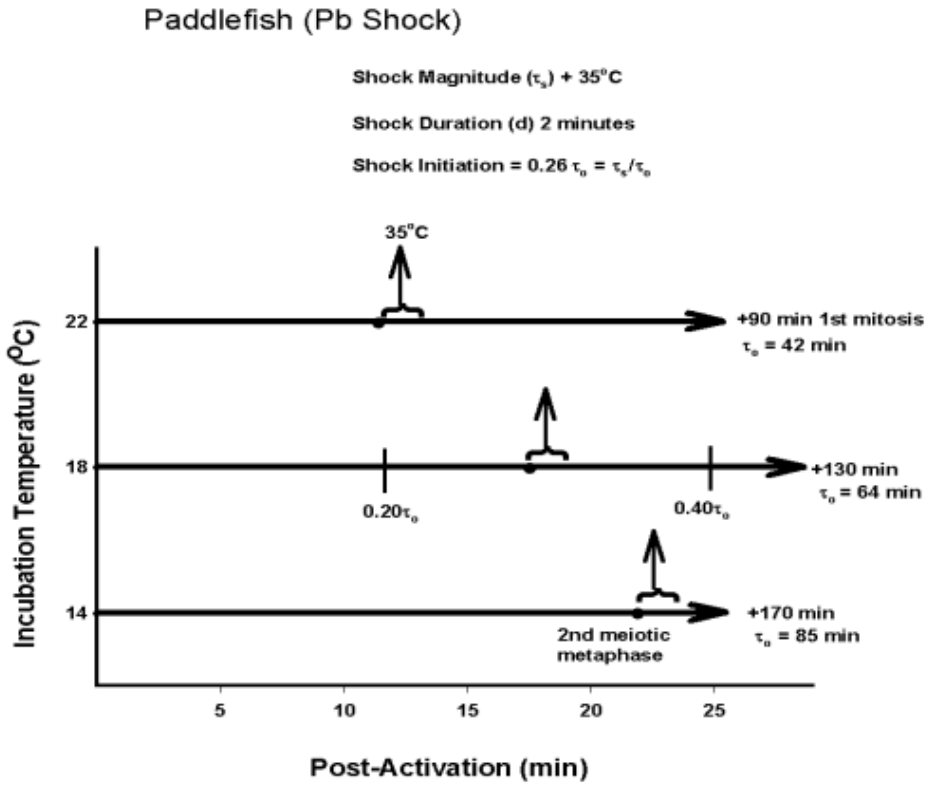

Fig. 5. Incubation temperature effect on Em shock time for gynogenetic induction in paddlefish. Data from Mims and Shelton (1998) and Mims et al. (2005).

1.5-1.9τ_o is the optimum time to initiate heat shock for endomitotic (Em) manipulation. Ploidy induction for grass carp has been most effective at 0.24-0.28τ_o for early shock (Pb) or 1.6-1.9τ_o for late shock (Cassani and Caton, 1985, 1986; Cassani et al., 1990). Shirak et al. (1998) described cytological development in tilapia zygotes relative to late shock gynogenesis. The pattern of cell division at 28°C correlated well with the time to first cleavage summarized by Shelton and Popma (2006). Induction was optimal during first mitotic spindle-fiber formation in metaphase (1.3-1.5τ_o or 0.6*T* at 28°C); metaphase at 28°C started at about 50 min (0.63T, or 1.6τ_o) post-insemination and the first furrow formation was about 80 min.

Effectiveness of shock times for Nile tilapia are displayed in Fig. 6; note that at 28°C , the optimal induction for endomitotic heat shock is earlier (0.8-0.9τ_o) than pressure shock (1.3-1.5τ_o), while cold shock is about 2.4τ_o. Palti et al. (1997) noted this same relationship in rainbow trout for both Pb (heat = 0.14τ_o vs pressure = 0.21-0.27τ_o) and endomitotic induction (heat = 0.9-1.1τ_o vs pressure = 1.5-1.9τ_o). Chourrout (1986) suggested that induction mechanisms might be different for shock types, e.g., disruption of spindle-fibers or interfering with furrow formation in first cleavage. In addition to rate of development, effectiveness of shock application is affected by asynchronous cell division. Time from the earliest cleavage initiation until the last in tilapia was dis-

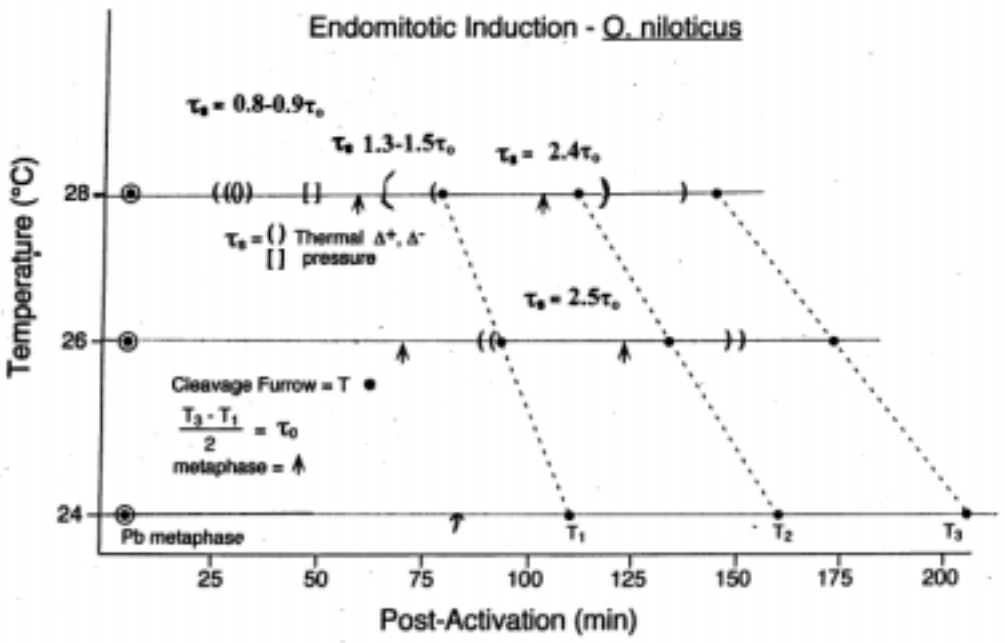

Fig. 6. Incubation temperature effect on Em shock time for gynogenetic induction in tilapia. Data from Hussain et al. (1991, 1998), Mair (1993), Hussain (1995), Shirak et al. (1998), and Shelton and Popma (2006).

persed over a 20-min period (Shirak et al., 1998). Whereas, duration of the first mitotic cleavage in rainbow trout was dispersed over 160 min at 10°C and initiation of cleavage varied by about 40 min between individuals from different populations (Hershberger and Hostuttler, 2005). Thus, induced diploidization at Em-gynogenesis is less likely to result in a uniformly narrow optimal effect compared to induction success at Pb-manipulation.

Reproductively limited fishes can be developed by various techniques, with differing complexities, and therefore involve various time constraints and/or degrees of security against possible reproduction. All-female progeny produced by gynogenesis was one of the first applications of ploidy manipulation in biological assessments (Stanley, 1976); no shock was used in the production of these gynogenotes for a large-scale study of monosex grass carp in Lake Conway, Florida. Only about 45,000 diploids were produced from over 58 million eggs; shock treatment obviously would have increased the incidence of polar body retention, but shock techniques were not well studied at the time. Gynogenesis can be an end (monosex) or a means to develop a monosex breeding system (Shelton, 1986). The total number of monosex fish needed for the Lake Conway study could have been produced ten-times over by using a single XX-neomale to fertilize eggs from only one grass carp female. The various options and interrelated sequence for integrating management of reproduction, illustrated in Fig. 3, is based on a model in which sex determination is considered to be characterized by homogametic (XX) females and heterogametic (XY) males. In the direct induction of monosex or sterile fish, the treatment can be developed rapidly but must be applied to each individual; absolute efficacy is rarely achieved as discussed relative to the inherent biological variability. However, induction can

be used as a means to develop broodstock (neomale) that would produce monosex offspring, if the presumptive discrete sex determination is effective.

The induction of triploids follows the protocol for gynogenesis with the exception that untreated sperm from conspecifics is used for activation so that the paternal genome is incorporated rather than using heterologous DNA-deactivated spermatozoa to initiate development. Triploidy is induced by early shock to retain the 2nd meiotic polar body. Verification of polyploidy requires progeny examination through karyotyping, red-blood cell nuclear analysis, or quantitative DNA determination (Benfey et al., 1984; Johnson et al., 1984). Triploidization has tremendous potential in the production of sterile fishes for water resource management or for assessment studies. The level of gonadal development differs in triploid males and females. In general, triploid fish have smaller gonads than diploids; males may have near normal-size testes, but with limited spermatogenesis, while ovaries of 3N fish are poorly developed and typically have few ova (Wolters et al., 1982; Allen et al., 1986; Johnson et al., 1986; Van Eenennaam et al., 1990; Piferrer et al., 1994; Tiwary et al., 2004).

The application of reproductively limited fish to aquaculture was a logical precursor to considering these techniques for exotic introductions but some of these control measures were developed and practiced only after introductions had occurred (Shelton, 1986). Sterile triploidy was first developed in grass carp in a serendipitous hybridization study (Marian and Krasznai, 1978). These techniques also have been tested with several other warmwater species (Mirza and Shelton, 1988; Rothbard and Shelton, 1993; Kerby et al., 1995; Rothbard et al., 2000) and salmonids (Donaldson and Hunter, 1985; Benfey, 1996).

Phenotypic Sex Reversal

Early efforts to manage unwanted reproduction in fishes focused on controlling recruitment for tilapias in aquaculture through monosexing (Guerrero, 1975; Shelton et al., 1978; Shelton, 1989; Wohlfarth and Hulata, 1989; Zohar, 1989; Phelps and Popma, 2000). Other applications relative to sexual dimorphism include the elimination of unsightly precocial salmonid males by all-female culture (Johnstone, 1996) or improved growth and reduction in recruitment for common carp (Gomelsky, 2003; Tzchori et al., 2004). Recent reviews provide an overview of these applications (Komen and Richter, 1993; McAndrew, 1993; Patino, 1997; Phelps and Popma, 2000). The assumptions are that (a) treatment must proceed during a critical period of gonadal differentiation, (b) steroids (androgens/estrogens) mimic natural induction by genetic sex-determining factors so as to alter development of the phenotypic or gonadal sex, (c) the exogenous steroid must be efficacious, adequately concentrated, and efficiently delivered so as to provide the physiological or pharmacological effect (Shelton et al., 1995), (d) steroid-induced development of gonadal sex does not spontaneously revert, and, (e) genotypic sex is not affected by the phenotypic alteration.

While there are deviations in detail for particular species or under certain circumstances, these generalized guidelines have resulted in the development of effective programs for sex control in many species of diverse phylogeny (Hunter and Donaldson, 1983; Wohlfarth and Hulata, 1983; Shelton, 1989; Dunham, 1990; Pandian and Sheela, 1995; Donaldson, 1996; Pandian and Kirankumar, 2003).

Studies of sex reversal for tilapias were initiated in the mid-1960s, underwent rapid experimental development in the 1970s and attained commercialized application during the 1980s (Rothbard et al., 1983). Control factors include the selection of steroid, concentration, mode of delivery, age or size of treatment initiation, duration, and treatment conditions (Shelton et al., 1978, 1981; Phelps and Popma, 2000). The appropriate concentration of an efficacious steroid must be delivered throughout the period of gonadal differentiation. The labile period of gonadal differentiation can be identified through histological means (Jensen and Shelton, 1983; Parmentier and Timmermans, 1985).

Hormone-treated feed has been the most practical means of delivery, and characterizing steroid concentration in feed would appear to be an appropriate means of expressing dose rate. However, the amount of treated feed consumed is a more accurate consideration. The dose rates can be easily adjusted, either by different steroid levels in the diet or by adjusting feeding rates. In actuality, the combination of the two provides a more meaningful expression of dosage (Pandian and Varadaraj, 1988) as the product of feeding rate and steroid concentration (µg/g body weight/d) approximates the Pharmacologically Effective Dosage (PED; Shelton et al., 1995). Steroid PED levels for sex reversal of tilapias have been in the range of 1.5-3 µg/g/d.

A less-controlled, but effective, steroid delivery can be achieved through intra-peritoneal implants for species such as grass carp and paddlefish in which gonadal differentiation occurs at a large size and for which feeding a hormone-treated diet is not an option (Shelton, 1986; Shelton and Mims, 2003). But factors that alter growth rate have a more critical effect on sex reversal since physiological processes such as gonadal differentiation are influenced by a balance between chronological age (time) and growth (size; Shelton et al., 1995). Thus, the "period" of treatment must take age and size, and therefore growth rate, into consideration; this "window" of opportunity for treatment optimization was conceptualized for tilapias (Shelton et al., 1978) and extended to common carp (Komen et al., 1989; Komen and Richter, 1993; Shelton et al., 1995) and grass carp (Shelton, 1986).

Delivery of the steroid with intraperitoneal implants is more complicated because the dose rate depends on a diminishing diffusion rate from the capsule relative to the increasing body mass of the treated fish. Sex reversal for grass carp based on size at treatment and diffusion rate from the steroid implants was about 1.2 µg/g body weight/d (Fig. 7). The initial fish size for treatment of grass carp is 5-10 g compared to 50-100 g for paddlefish. Treatment effectiveness for paddlefish was improved when two 5-mg implants were used instead of one (Shelton, 1986; Mims et al., 1995).

Functional sex reversal can be considered a programmatic component, rather than a direct means of producing monosex fish. The genetic basis for phenotypic sex development is determined at fertilization and usually directs gonadal sex. But genetic regulation is more complicated than simple Mendelian inheritance would suggest (Shelton et al., 1983, Lester et al., 1989; Wohlfarth and Widekind, 1991; Trombka and Avtalion, 1993; Müller-Belecke and Hörstgen-Schwark, 1995; Tuan et al., 1999). Postulated breeding programs for tilapia (Shelton et al., 1978) have been applied (Mair et al., 1997), but usually with some non-uniformity. However, all-female rainbow trout culture using sex-reversal and breeding has been effectively practiced on a commercial scale (Johnstone, 1996).

Premeiotic germ cells have sexual bipotentiality and they can be exogenously influenced by various environmental factors as demonstrated by steroid-induced sex reversal and temperature-affected sex ratio modification. Treatments for tilapias through oral delivery can be 95-100% effective, but those for grass carp and paddlefish with hormone implants have usually been 90% or less. These ploidy manipulation and sex-reversal protocols can be used in developing broodstock for breeding monosex progeny (Fig. 3), or for direct production of monosex or triploid populations. Pertinent physiological factors must be considered for manipulation efficiency.

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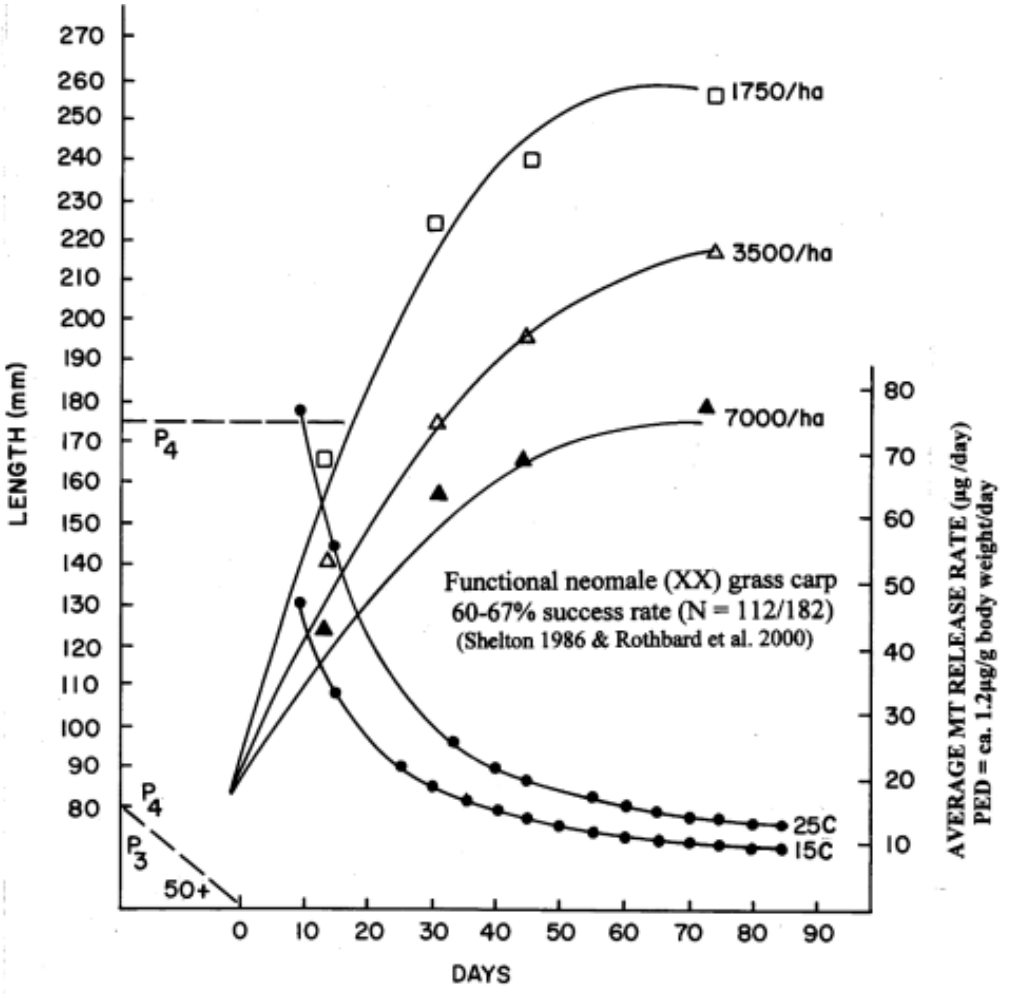

Fig. 7. Growth and methyl testosterone release from intraperitoneal implants for sex reversal of grass carp (Shelton, 1986). Gonadal differentiation stages (Shelton et al., 1995): P3 = morphological, P4 = cytological.

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