MALE GERM CELL PROLIFERATION IN FISH UNDER THE INFLUENCE OF VARIOUS FACTORS WITH SPECIAL REFERENCE TO INDIAN MAJOR CARPS

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The end product of germ cell maturation is the spermatozoa that are produced through a definite pattern in nature that requires a special environment. During proliferation of diploid germ cells, the spermatogonia repopulate the testis lobules by mitotic cell division. The natural germ cell proliferation and maturation has been modulated and manipulated by various factors to get maximum viable sperms for aquaculture practice. Environment and chemicals of natural and synthetic origin can interact with the endocrine system and alter the male germ cell generation and proliferation/inhibition in fish. In the present investigative review, an attempt has been made to ascertain various factors that influence the spermatogonial proliferation and maturation of fish with a special reference to Indian major carps, *viz.* catla, *Catla catla,* rohu, *Labeo rohita and,* mrigal, *Cirrhinus mrigala*. It was observed that physical factors such as temperature and photoperiod, and the chemical stimulants such as clomephene citrate, hormones and other chemicals stimulate the process. However, if not applied in optimal conditions/doses it may retract the germ cell proliferation and the entire spermatogenesis.

INTRODUCTION

Male germ cell (GC) proliferation that includes spermatogenesis is a dynamic process that follows a definite and specific pattern in nature and ultimately produces haploid spermatozoa. GCs are considered to be immortal since they create a link between generations and multiply through mitosis. The cellular basis of reproduction in males is the spermatozoon. They develop from testicular germinal epithelium (Grier and Taylor, 1998) (spermatogonial stem cells), which first proliferate (spermatogonia), then differentiate into spermatocytes that undergo meiosis to become haploid spermatids that differentiate to become spermatozoa (spermiogenesis). The gonadal function changes during annual reproductive cycles in male fish. During proliferation of diploid germ cells, the spermatogonia repopulate the testis lobules by mitotic cell division.

Though GC proliferation and spermatogenesis is a natural dynamic process, it has been modulated and manipulated by various factors to get maximum viable sperms for aquaculture practice. Environment and chemicals of natural and synthetic origin can interact with the endocrine system and alter the male germ cell generation and proliferation/ inhibition in fish. Various workers reported sensitiveness of spermatogenesis process by

environmental factors such as temperature, photoperiod etc. (Nobrega *et al.*, 2009; Batlouni *et al.*, 2009; Sarkar *et al.*, 2010). The induction of spermatogenesis by different GnRHa delivery system enhanced spermiation for longer period in cultured fishes (Mylonas and Zohar, 2001). The duration of spermatogenesis is also influenced by temperature (Nobrega *et al.,* 2009). The evaluation and manipulation of environmental factors on reproduction provides valuable information for aquaculture and species management (Schulz *et al.,* 2005). In the present investigative review, an attempt has been made to ascertain various factors that influence the GC/spermatogonial proliferation and maturation of fish with a special reference to Indian major carps (*Catla catla, Labeo rohita, Cirrhinus mrigala and Labeo calbasu)*.

MATERIALS AND METHODS

Indian major carps having differ ent maturity stages were sampled to estimate their testis somatic index (TSI) at different times of the year. The two year old fishes were subjected to several external factors (environment) and chemical inducers (hormones) and other inducing agents to estimate the semen quantity and quality, and assess the male GC/spermatogonial cell proliferation over a period of time (after every 2 weeks) and also the regulation of spermatogenesis annually. A comparative evaluation of male GC proliferative/ spermatogenic cycles with other species has also been done. To check the effect of different factors, the fishes were reared at different water temperatures (20 °C, 24 °C, 28 °C, 32 °C, 36 °C), at varying photoperiods (14 h D: 10 h L, 12 h D: 12 h L, 10 h D: 14 h L), priming of hormones to males one month prior to spawning season and during season (0.1ml Kg^{-1} body weight) and administration of a chemical clomiphene citrate (5, 4, 3, 2 mg Kg^{-1} body weight for 3 weeks). Each treatment was sampled every 15 days interval to evaluate their TSI, sperm yield (ml Kg^{-1}), sperm count, spermatocrit value.

For isolation of GC, adult male fish (exposed/treated and control) were anaesthetized prior to dissection with 1–2 phenoxyethanol. Anaesthetized fish were taken out of the water and dissected ventrally, and gonad (testis) was taken out by cutting their basal stalk. The collected gonads were cut into pieces of 2–3 mm size using sterilized scissors. These small pieces were separated to get single cells using 2% collagenase (Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle'emdium/Ham F-12 medium (DMEM/F-12 Sigma, St. Louis, MO) at 25 ºC. The cell suspension was incubated with 0.25% trypsin/1mM EDTA and 0.03% DNase I for 30 mins under similar conditions. An equal volume of fetal bovine serum (FBS, Gibco, Waltham, MA, USA 02451) was used to deactivate trypsin. A 45 µm mesh cell strainer was used to filter the cell suspension and centrifuged at 200 g for 10 min and re-suspended in DMEM/F-12. Thereafter, germ cell isolation was done by percoll (MP Biomedicals, LLC, France) gradient centrifugation that comprised centrifuging testicular cells for 10 min (800 g) at 25 °C. The process resulted in the appearance of three distinctive bands. The phase containing the principal cells (GCs) was harvested from the middle layer, rinsed and subjected to trypan blue (0.4%) dye exclusion test for cell viability and cell counting was done following standard methods.

GC concentration in the gonad of each fish was estimated by counting the number of cells diluted in PBS (dilution 1:10) using a haemocytometer at 100 X magnification and a microscope. The number of GCs was counted in 20 squares of the cell chamber with two repetitions, and GC content was expressed as the absolute number of GCs obtained from the gonad of each fish by multiplying the number of GCs by the volume of each GC sample (0.5 ml).

The data were analyzed using students t-test and a P value of $P < 0.05$ was taken as significant.

RESULTS AND DISCUSSION

Fish species inhabiting diverse aquatic ecological systems offers an enormous challenge to generalize their reproductive fitness and characters that includes their sexual dimorphism, male germ cell spermatogenic cycle (*in vivo and in vitro*), spermatozoon structure and the external inducers that modulate these in fish. It varies with class and even varies with the Infraclass teleosts e.g. freshwater fish to marine fish also. Here our focus species are from freshwater systems with particular reference to Indian major carps (IMCs). The testes in most teleosts reported including that of the IMCs are bilobed, with spermatogonia dispersed throughout the gametogenic epithelium of the seminiferous tubules. The sexual dimorphism is clear in species during the reproductive season (perennial spawners in nature), however, some are very inconspicuous during non-spawning season (IMCs) (Routray *et al.,* 2009; Verma *et al*., 2009). The literature on the annual reproductive cycle in fish has demonstrated variations in germ cell and testicular somatic cell proliferation during the year (Chaves-Pozo *et al*., 2007; Mc Clusky, 2005). The manipulation of reproduction and spawning time through photoperiod has been reported in *Heteropneustes fossilis* (Sundararaj and Seghal, 1970), *Clarias batrachus* (Singh and Joy, 1998; Acharia *et al*., 2000) and *Cirrhinus reba* (Verghese, 1975). Among environmental factors, temperature plays an important role as a modulator of fish reproduction, including those aspects related to testicular function and spermatogenesis (Billard, 1986; Quintana *et al*., 2004).

The factors that were found to modulate the TSI, germ cell proliferation and subsequent milt yield in carps included physical factors and chemicals such as water temperature (28 °C), photoperiod regime (10h Dark: 14h Light), priming with GnRH inducement one month prior to spawning season and during season (0.1 ml Kg^{-1} body weight) and clomiphene citrate (4 mg Kg^{-1} body weight for 3 weeks) (Fig. 1, 2, 3)**.** The TSI and the milt yield are the direct consequences of spermatogenesis in fish. The collected milt was analyzed for its motility, count and spermatocrit values and all these parameters were not affected by the modulators used for spermatogenesis and the final milt yield in carps that were similar to other fish. Various workers reported the role of temperature in altering the duration of spermatogenesis in teleosts (Egami and Hyodo-Taguchi, 1967; Vilela, 2003). For instance, the spermatogenic process was faster in Nile tilapias kept at higher temperatures (30 °C – 35 °C) compared with tilapias kept at 25 °C, which was considered the physiological temperature for reproduction in this species (Vilela, 2003; Lacerda 2006). It

was also observed in the present study that if the conditions applied are not optimal for the male brood fish, the germ cell proliferation during spermatogenic cycle (starting from testicular growth to spermatozoa production) gets affected and resulted in low milt yield. The germ cell numbers also increased significantly when the rohu and catla were reared at a photo period regime of 10hD:14hL. Similarly, the treatment of clomiphene citrate to male catla and rohu also resulted in significant increase in the GC of both the species (Fig.4). The spermatogenic cycle varied among the fish species (Table-1) and also the sperm density (40 to 53,000 million ml^{-1}). The spermatogenic cycle cannot be generalized in fishes as the requirement to attain maturity and sperm production varies among species. The influences of Photoperiod and temperature on spermiation were demonstrated in *Cyprinus carpio* (Davies *et al.*, 1986), *Carasius auratus* (Kawamura and Hanyu, 1950).

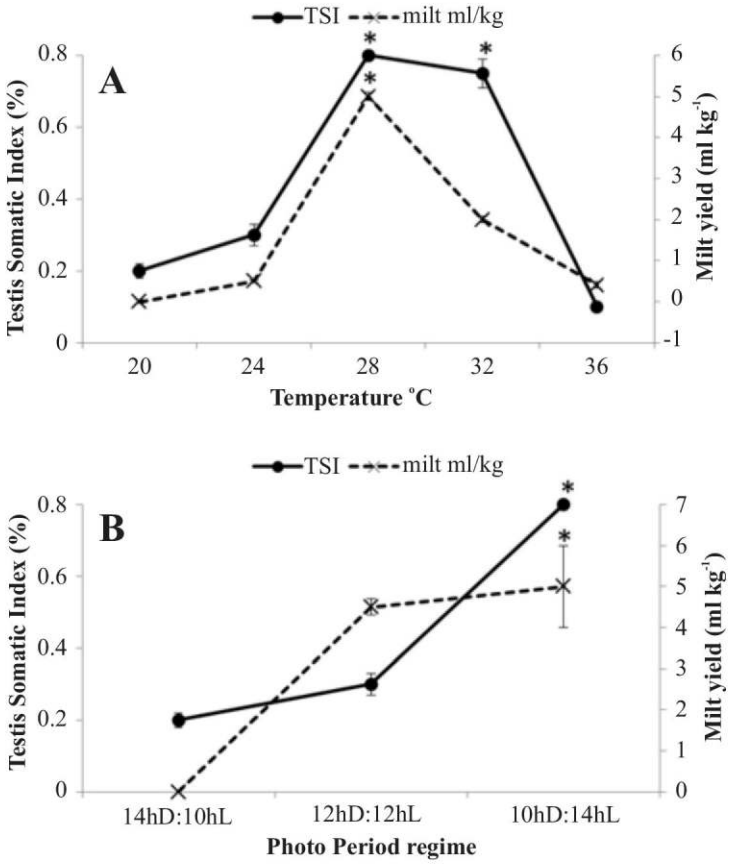

Fig. 1. Effect of physical factors on the milt yield and TSI of *L. rohita* during the month of January-February (winter months). A: water temperature; B: photoperiod regime. Data shown as mean value ± SE (n=60), asterisks indicate significant values ($P < 0.05$).

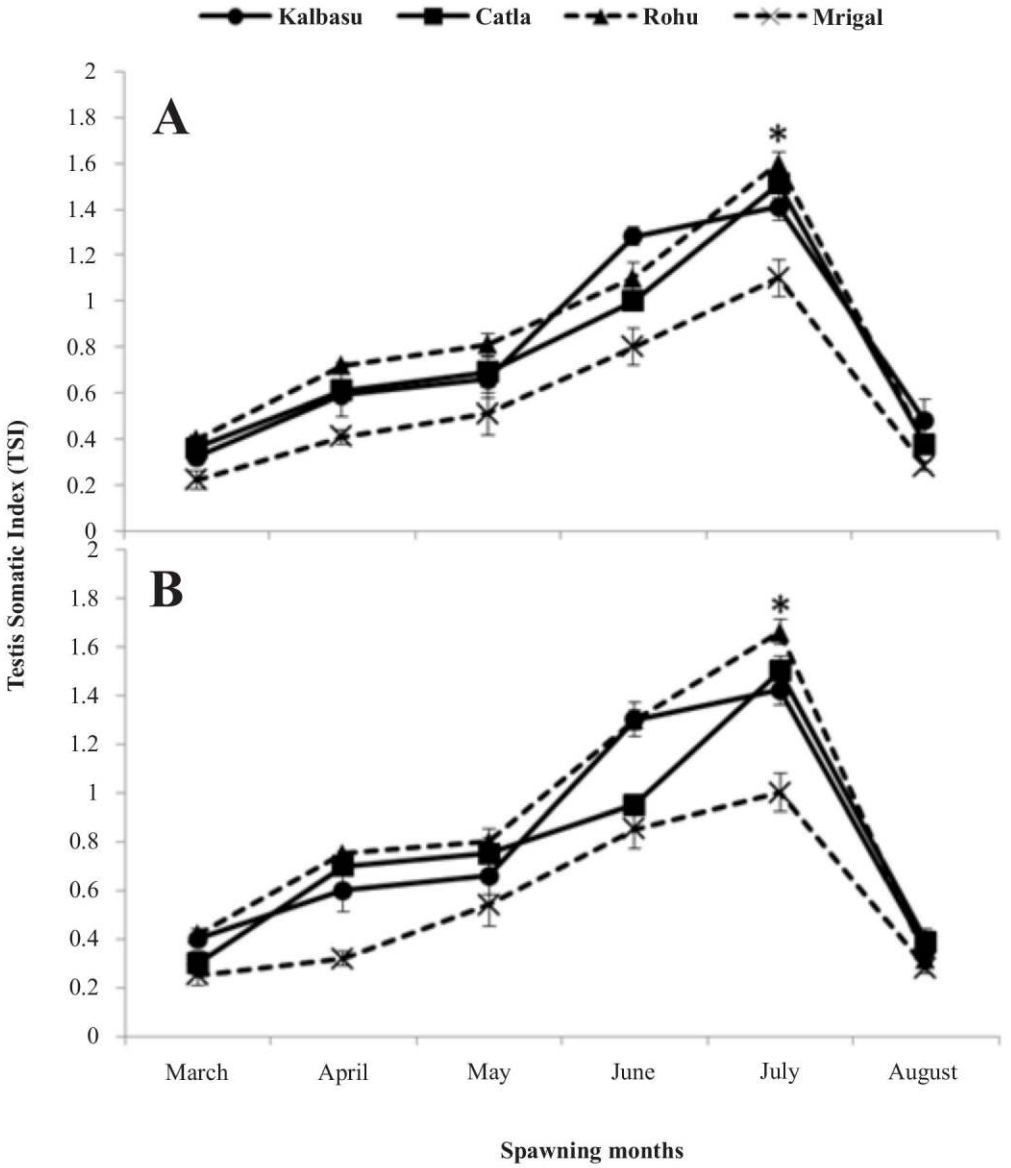

Fig. 2. Changes in the TSI of IMCs and *Labeo calbasu* during spawning season. A: Normal seasonal change and B: GnRH induced change. Data shown as mean value ± SE (n=36), asterisks indicate significant values ($P < 0.05$).

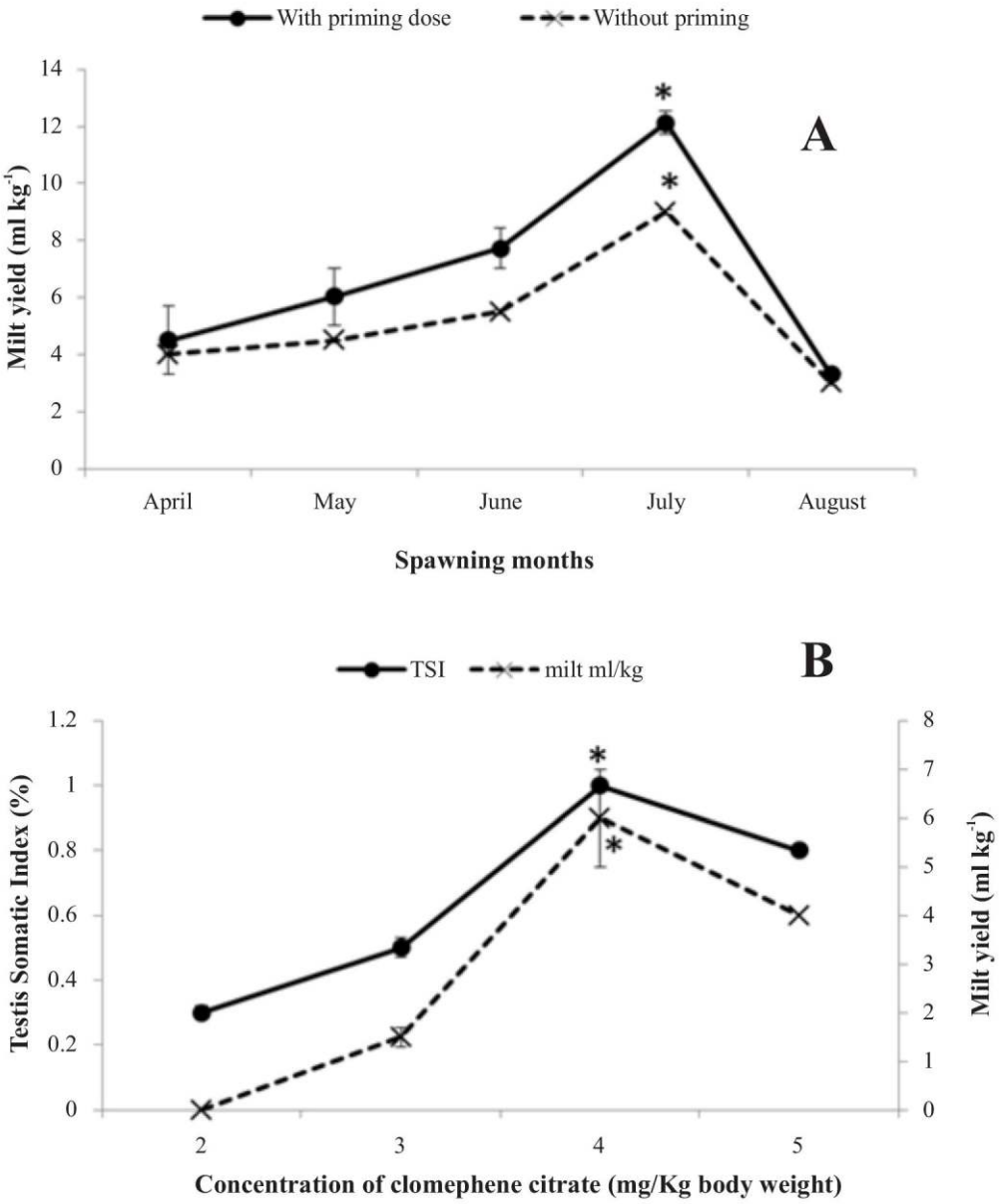

Fig. 3. A: Effect of GnRH priming of brood fish (15 days prior to milt collection) on milt yield of *Labeo rohita* during different months of the spawning season. B: Effect of clomephene citrate priming on the milt yield and TSI. Data shown as mean value ± SE (n=60), asterisks indicate significant values ($P < 0.05$).

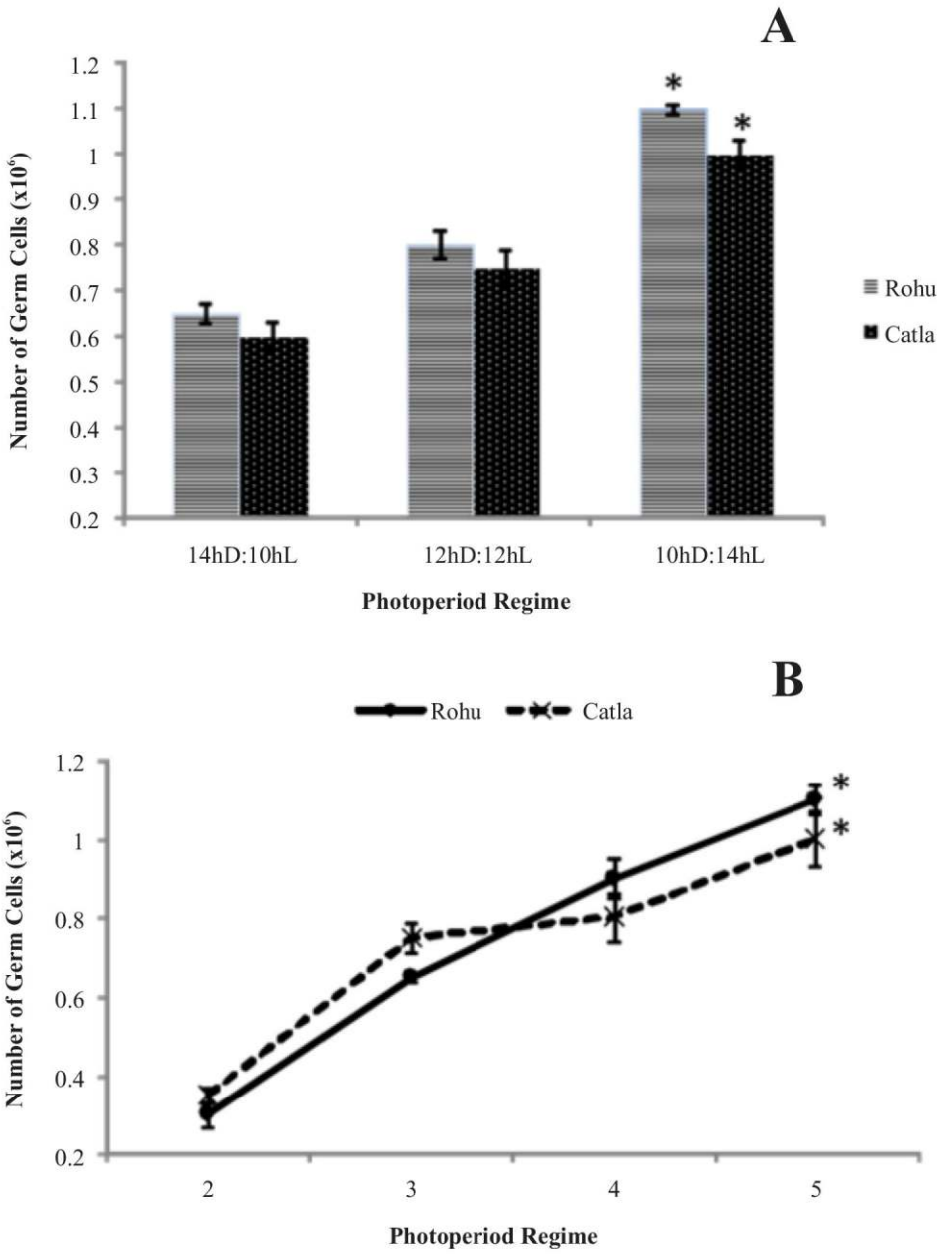

Fig. 4. A: Effect of photoperiod (A) and clomephene citrate treatment (B) on the germ cell proliferation/depletion level of rohu, *Labeo rohita* and catla, *Catla catla*. Data shown as mean value ± SE (n=60), asterisks indicate significant values ($P < 0.05$).

Here, the IMCs and the Kalbsu had a similar trend of spermatogenic cycle in natural cycle but when intervened with modulators such as temperature, photoperiod, hormonal priming and chemical priming, this could be altered to meet the demands of aquaculture. A similar approach may be useful for other species also. With the advancements in reproductive biology of fishes, it is now possible, that spermatogenesis can be modulated and manipulated by various factors to get maximum viable sperms for aquaculture practice.

Table 1: A comparative account of gonadal (spermatogenic cycle) in different fishes

Species	Common name	Spermatogenesis	Inducement	Reference
Heteropneustes fossilis	Catfish	Annual gonadal cycle.	Natural cycle	Sundararaj and Vasal (1976)
S. gairdneri	Rainbow trout	Twice in year (Autumn and summer)	Natural cycle	Billard and Breton (1978)
S. gairdneri	Rainbow trout	Advanced maturation and spawning by 6-12 weeks at 9°C	Photo period manipulation	Whitehead *et al.* (1978)
Oreochromis niloticus niloticus	Tilapia	Testicular activity restricted to autumn and winter, and testes are in resting phase from July-September in equatorial zone.	Natural photoperiod	Hyder (1970)
Indian major carps	Catla, Rohu and Mrigal	Increasing gonadal recrudescence; males attain maturity earlier than females	Photo period manipulation	Verghese (1975)
Indian major carps	Catla, Rohu and Mrigal	Advancement of sexual maturation and off-season spawning of IMCs	Photo thermal manipulation	Sarkar *et al.* (2010)
Danio rerio	Zebra fish	Combined duration of meiotic and spermiogenic phases is very short in this species and lasts approximately 6 days	Natural cycle	Marcelo *et al.* (2009)
C. fasciata	Costa Rican tiger rump	Spermatogenic phase 6 days.	Natural cycle	Sinha *et al.* (1979)
P. sphenops	Black molly	Spermatogenic phase 21days	Natural cycle	Felice and Rash (1969)
L. calbasu	*Medium carp*	Spermatogenic phase 15-21 days	GnRH based inducement	Present study
Abramis brama	Bream	Early Gonadal maturation after inducement.	Induced with PiT, hCG	Kucharczyk *et al.* (1997)

Anguilla japonica	Japanese eel	Achieved spermiation within 3 months after inducement	Induced with PiT, hCG	Miura *et al*. (1991)
Argyrosomus hololepidotus.	Mulloway	Gonadal maturation achieved with induction	Induced with hCG	Battaglene and Talbot (1994)
Macquaria novemaculeata	Australian bass	Gonadal maturation achieved with induction	Induced with hCG	Battaglene and Selosse (1996)
Micropterus salmoides	Largemouth bass	Gonadal maturation achieved with induction	Induced with hCG	Mayes *et al.* (1993)
Perca fluviatilis	Perch	Natural spawning from early spring i.e in August and ends later in the autumn	Natural cycle	Malison *et al.* (1986)
Gadus morhua	Atlantic Cod	Spermatogonial proliferation starts in August to February)	Natural Reproduction	Fernanda *et al.* (2008)
IMCs & *L. calbasu*	Catla, Rohu Mrigal, Kalbasu	Spermatogenic phase 15-21 days	GnRH based inducement	Present study
IMCs	Catla, Rohu Mrigal	Spermatogenic phase 30-45 days	Longer photo period (>12h) and optimal temperature	Present study
IMCs	Catla, Rohu Mrigal	Spermatogenic phase 15-20 days	Clomephene citrate	Present study

Abbreviations used in table: hCG Human chorionic gonadotropin; ground pituitaries or pituitary extracts of vertebrate or piscine origin PiT

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