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Spawning Induction in the Carp: Past Experience and Future Prospects - A Review

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Abstract

Most fish in aquaculture either fail to breed in captivity or their spawning occurs sporadically and late in the season. This is mainly due to the lack of natural cues in captivity, which leads to dysfunction of the endocrine axis regulating oocyte maturation and ovulation. Hypophysation as a remedy for this situation in fish has been employed in aquaculture since the 1930s and is still widely practiced. However, using crude pituitary homogenates in local hatcheries has frequently ended in failures that were attributed to the inconsistent potency of the injected material and the unknown ovarian stage of the recipient fish. Since the mid 1980s, hypophysation has improved through the introduction of a standardized dry carp pituitary extract in which

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the luteinizing hormone (LH) content and activity have been calibrated (calibrated carp pituitary extract = CCPE). Induction of spawning, however, is successful mainly in female cohorts in which 65% or more of the oocytes in an ovarian biopsy have migrating germinal vesicles. Further, due to decreasing quantities of industry-processed common carp and the expansion of ornamental carp production (koi and goldfish), the growing demand for CCPE could not be met, and an alternative had to be found. A hypothalamic approach, introduced into Israeli aquaculture in 1993 (called Dagin), combines a superactive analog of sGnRH (10 μg/kg), with the water-soluble dopamine (D_2) receptor antagonist, metoclopramide (20 mg/kg). The progress of oocyte maturation in ovarian biopsies has been studied in parallel with changes in levels of LH, estradiol, and the maturation-inducing steroid (MIS; 17α, 20β, dihydroxy-4-pregnene-3-one). The hormone profile indicated that the gradual increases in LH and MIS following a single administration of Dagin were similar to those in fish treated with priming and resolving doses of CCPE. This would explain why Dagin is effective even when only a single injection is given, saving labor and reducing handling stress. CCPE and Dagin were tested in parallel on common carp in a commercial hatchery. The spawning ratio and embryo viability were similar, although the latency between injection and ovulation was considerably longer and more variable in Dagin-treated than in CCPE-treated carp. It is recommended to use CCPE at the beginning and end of the spawning season when the LH content in the pituitary is low, and Dagin in mid-season and in field spawning. Future prospects raise the possibility that by employing molecular tools, a recombinant carp LH will be produced that will have the regular and expected potency of the hypophyseal approach without the risk of spreading pathogens from donor fish to broodstock. Work along this line is currently in progress.

Introduction

The common carp is one of the most important species in aquaculture, with an annual global production of over 22 million metric tons comprising, in 2002, nearly 14% of the total freshwater aquaculture production. Seventy percent of this is produced in China. Common carp production increased by an average global rate of 9.5%/yr between 1985 and 2002 (FAO, 2008). Viable aquaculture of a fish species, including carp, requires control over its entire life cycle beginning with reproduction. Such control enables a steady supply of fish for stocking in grow-out ponds both during and out of season, and allows genetic improvement of the cultured fish for better growth, disease resistance, and meat quality.

Certain fishes, such as carp, readily reproduce in captivity, but spawning occurs late in the season and is not synchronized. Most cultured fish, however, do not reproduce at all under captive conditions due to dysfunction of one or more sites along the brain-pituitary-gonadal (BPG) axis. Most commonly, females fail to undergo follicular maturation and ovulation, while captive males may produce milt, but the amount will not be large (Zohar and Mylonas, 2001).

In some cases, it is sufficient to manipulate environmental factors such as temperature, photoperiod, salinity, tank size, or substrate to elicit normal spawning. In others, exposure of the females to the male courting behavior and pheromones has a similar effect (Kobayashi et al., 2002). However, in many fishes, hormonal manipulation is required for the induction of ovulation and spawning. Male carp do not require hormonal treatment to complete spermatogenesis but treatment is given to them (generally half the dose given to females) to increase milt volume. Therefore, emphasis is given here to female reproductive biology.

Endocrine Axis Regulating Reproduction in Females

To understand the endocrine framework that is manipulated by hormonal induction of spawning, a brief account is presented here of the normal sequence of endocrine changes in relationship to the progress of oocyte maturation, ovulation, and egg release.

In subtropical countries such as Israel, carp attain sexual puberty at less than one year and a body weight under one kg (Sarig, 1966). At puberty, the ovary contains oocytes that are in the process of growth, together with smaller oocytes that have dark cytoplasm and are destined to develop in following seasons (Levavi-Zermonsky and Yaron, 1986). Pubertal carp males can be recognized by milt that oozes from the genital papilla upon application of gentle pressure on the abdomen.

As the oocyte starts growing during fish puberty or during the annual recrudescence, it is arrested at the prophase of the first meiotic division. While still at this chromosomal stage, the oocyte becomes vitellogenic, i.e., it sequesters from the circulation and accumulates yolk (vitellogenins) and chorionic (choriogenins) proteins produced by the liver under estradiol stimulation. The oocyte reaches its final diameter of about 1 mm and is then considered post-vitellogenic (Fig. 1).

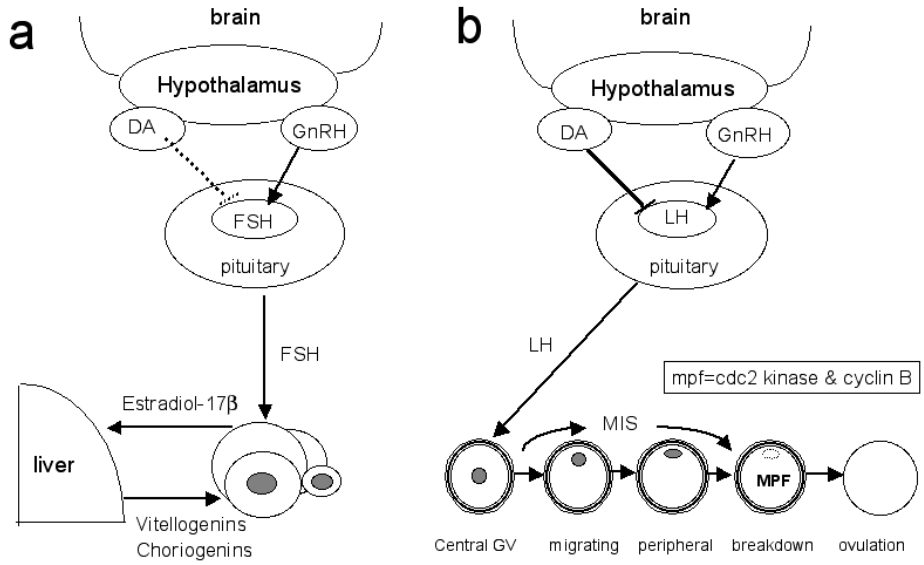

Fig. 1. The main endocrine factors regulating reproduction in (a) female fish during vitellogenesis and (b) post-vitellogenic females during final oocyte maturation and ovulation. During vitellogenesis, gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus stimulates the secretion of FSH that, in turn, accelerates the secretion of estradiol from ovarian follicles. Meanwhile dopamine (DA), also secreted by the hypothalamus, can antagonize the GnRH effect. In the liver, estradiol promotes synthesis and secretion of vitellogenins and choriogenins into the circulation. These are then sequestered by the oocyte to form the yolk and chorion. In post-vitellogenic females, GnRH stimulates the synthesis and release of luteinizing hormone (LH) from the pituitary gland, while DA can antagonize this effect. LH stimulates secretion of maturation-inducing steroid (MIS; 17α, 20β-DHP) from the ovarian follicle, which induces formation in the oocyte of the maturation-promoting factor (MPF). MPF, consisting of cdc2 kinase and cyclin B, supports the resumption of meiosis and subsequent ovulation (modified from Yaron and Sivan, 2006).

The post-vitellogenic oocyte may remain quiescent for several months. It will enter the process of final maturation, i.e., the resumption of meiosis, in response to a surge of luteinizing hormone (LH) following environmental cues (temperature, photoperiod, water flow, etc.) or social and pheromonal stimuli. When LH binds to its receptor on granulosa cells, the ovarian follicle starts to mature, manifested by (a) the production of maturation-inducing steroid (MIS), (b) acquisition of oocyte maturational competence (OMC), (c) formation of the maturation-promoting factor (MPF) and resumption of meiosis, and (d) cytoplasmic maturation involving changes in yolk proteins and lipids.

These steps are followed by ovulation, i.e., rupture of the follicle and release of the eggs into the ovarian lumen. The process of oocyte maturation is reflected morphologically by the migration of the germinal vesicle (GV) toward the animal pole (GV migration) and its disappearance, a stage known as GV breakdown (GVBD). The latter indicates completion of the first meiotic prophase. Chromosomes then condense, a spindle is formed, and extrusion of the first polar body marks the end of the first meiotic division (reviewed by Yoshikuni and Nagahama, 1991). At this stage the egg is ovulated, but meiosis is arrested again in metaphase II. Completion of the second meiotic division and extrusion of the second polar body are delayed until the egg is stimulated by the penetration of a spermatozoon (reviewed by Nagahama and Yamashita, 2008); the egg is thus diploid (2N) at fertilization.

It is suggested that, in the wild, pheromones such as 17α, 20β-dihydroxy-4-pregnen-3-one 17 (DHP) secreted by sexually active males stimulate ovulation in post-vitellogenic females (Kobayashi et al., 2002). This is probably due to a surge in circulating LH (cGtH or GTH II), which may reach levels above 200 ng/ml (Zhao et al., 1984; Levavi-Zermonsky and Yaron, 1986; Santos et al., 1986; Drori et al., 1994). This surge is attributed to an increase in the release of GnRH from hypothalamic nerve endings ramifying within the rostral pituitary (rostral pars distalis), possibly associated with decreased dopaminergic inhibitory tonus. The decrease in dopaminergic inhibition may result from changes in catecholamine levels and turnover in the brain and pituitary (e.g., Peter et al., 1986; Saligaut et al., 1992; Senthilkumaran and Joy, 1995) or changes in the expression level of the dopamine D2 receptor which is dependent on estradiol levels (Levavi-Sivan et al., 2003, 2005, 2006). Either way, this will augment the effect of GnRH on LH release.

The surge in LH stimulates the secretion of MIS from the ovarian follicles. MIS of the common carp has been identified as 17α, 20β-dihydroxy-4-pregnen-3-one (DHP; Jalabert et al., 1977; Epler, 1981a,b; Scott et al., 1982; Yaron and Levavi-Zermonsky, 1986; Kime et al., 1987; Nagahama, 1987; reviewed by Nagahama, 1997). Receptors for MIS develop in the plasma membrane of oocytes under LH stimulation (reviewed by Nagahama and Yamashita, 2008), which may explain why the capacity of oocytes to respond to MIS develops only following the LH surge (Kime et al., 1987). The binding of MIS to its membrane receptors is followed by formation of the maturation-promoting factor (MPF), a complex consisting of cdc2 kinase and cyclin B (Yamashita et al., 1992, reviewed by Nagahama et al., 1993). The components of MPF are involved in the process of cell cycling, including resumption of meiotic division. (Nagahama, 1997; reviewed by Nagahama and Yamashita, 2008).

Hormonal intervention in the processes described above is possible in at least two ways. One approach is to create an artificial gonadotropin surge by administration of a gonadotropic preparation (heterologous gonadotropin such as the human chorionic gonadotropin, LH-containing carp pituitary homogenate or extract, or recombinant gonadotropins). This is the hypophyseal approach or hypophysation. The alternative hypothalamic approach employs the hypothalamic peptide gonadotropin-releasing hormone (GnRH) or its superactive analogs (GnRHa), with or without a dopamine antagonist, to elicit a similar surge by stimulating the synthesis and release of endogenous LH.

The Hypophyseal Approach using Human Chorionic Gonadotropin (hCG)

Hypophysation as a technique for spawning induction in fish was introduced into aquaculture in the 1930s (Houssay, 1930; Von Ihering, 1937). Fresh pituitaries from homologous or heterologous species are homogenized and injected into fish. This method is employed in various countries using homologous pituitaries of fish processed in factories or glands excised from trash fish (e.g., Perdikaris et al., 2007).

One of the commercially available gonadotropins is the human chorionic gonadotropin (hCG) extracted from the urine of pregnant women. This heterologous gonadotropin has been successfully used as a spawn-inducing agent in many fishes, mainly marine species. Although potent in a variety of cases, and in spite of its purity and known standard potency, hCG, as a large glycoprotein, may elicit an immune response due to its heterologous source and may render recipient fish refractory to subsequent treatments. The importance of such alleged immunity is controversial (Van Der Kraak et al., 1989; Zohar and Mylonas, 2001; Levavi-Sivan et al., 2004). The generation of an immune response is probably dependent on the studied species or method used to determine the response. Nevertheless, in certain cyprinids including the common carp, hCG is only marginally effective in inducing ovulation, requiring repeated injections of relatively large doses and, occasionally, addition of a pituitary extract (e.g., Kucharczyk et al., 1997). Our own experiment with local common carp showed that even at doses above 2,000 IU/kg BW, hCG resulted in very poor spawning response (Table 1).

In Israeli aquaculture the previously-used protocol specified that glands taken for induction of spawning in a recipient fish weighing 1 kg should be taken from a female carp of similar weight during the breeding season. The glands were stored in absolute ethanol at room temperature until used (Rothbard, 1981). However, failures were frequent, attributed to inconsistent potency of the injected material and/or the unknown stage of the recipient fish. The following account reviews developments in the hypophyseal approach that have resulted in improved spawning induction in Israeli cyprinid aquaculture.

The Hypophyseal Approach using Calibrated Carp Pituitary Extract (CCPE)

A project was undertaken in the early 1980s to establish a biotechnical methodology for the commercial production of a hypophyseal preparation with calibrated biological potency suitable for spawning induction in carp species. Basic studies in our laboratory on the nature of gonadotropic stimulation of estradiol secretion from ovarian fragments of tilapia yielded a sensitive *in vitro* system that could be used to measure the gonadotropic potency of samples as small as 1:1000 of a carp pituitary (Yaron et al., 1982, 1985; Bogomolnaya and Yaron, 1984). This *in vitro* bioassay became instrumental for the project.

Table 1. Spawning experiment comparing CCPE (LH calibrated carp pituitary extract) and hCG in the Gan Shmuel hatchery, Israel, August 2005. Priming injection was given at 11:00 and the resolving dose at 23:00. Other details are as in Levavi-Zermonsky and Yaron (1986).

Group	*n*	*Priming dose*	*Resolving dose*	*Spawned*
1	8	CCPE 20%	CCPE 80%	8/8
2	8	hCG 400 IU	hCG 1100 IU	0/8
3	8	hCG 1000	hCG 2000 IU	1/8
4	8	–	hCG 2000 IU	1/8

Before embarking on a large-scale operation involving the fish-processing industry, the following biological and technical points had to be clarified:

(a) *the optimal season for harvesting pituitaries and the optimal size of donor fish.* Pituitaries were excised from male and female carp randomly collected at monthly intervals from the commercial harvest of Kibbutz Gan Shmuel. The content of cGtH (carp LH) was determined first by the *in vitro* bioassay and later by radioimmunoassay (RIA) according to Levavi-Zermonsky and Yaron (1986). The study revealed two peaks of pituitary LH content, one during the spring in March-May and the other during the autumn in October (Yaron et al., 1984; Yaron and Levavi-Zermonsky, 1986). LH content in the pituitary was higher in fish larger than 1 kg BW than in smaller fish of 0.3-0.99 kg BW (Fig. 2). Based on these findings, it was advised to harvest pituitaries from carp larger than 1 kg BW during April-May or September-October.

(b) *post-mortem harvesting*. To enable flexibility of the factory work force, it was essential to determine how soon the pituitaries should be excised following decapitation of the fish. Biological potency was determined in pituitaries excised from freshly decapitated carp heads and from heads stored at 4°C for 24 or 48 h. The results indicated that no considerable deterioration of the biological potency takes place in glands stored for 24 h at 4°C as compared with fresh glands (Fig. 3).

(c) *storage of glands and preservation of their biological activity.* To determine how to preserve biological potency of harvested pituitaries until extraction, excised glands were placed directly into absolute ethanol at room temperature (23°C) and stored for 2 months at 4°C or -20°C following several changes of ethanol. Other glands were frozen immediately after removal and stored at -70°C. Biological potency was tested as above and compared with standard carp GtH (obtained from Dr. E. Burzawa Gerard, Paris). Storage of glands in ethanol at 23°C or 4°C resulted in considerable loss of biological activity, while storage of glands in absolute ethanol at -20°C preserved biological activity at a level similar to that of glands frozen immediately and stored at -70°C (Fig. 4).

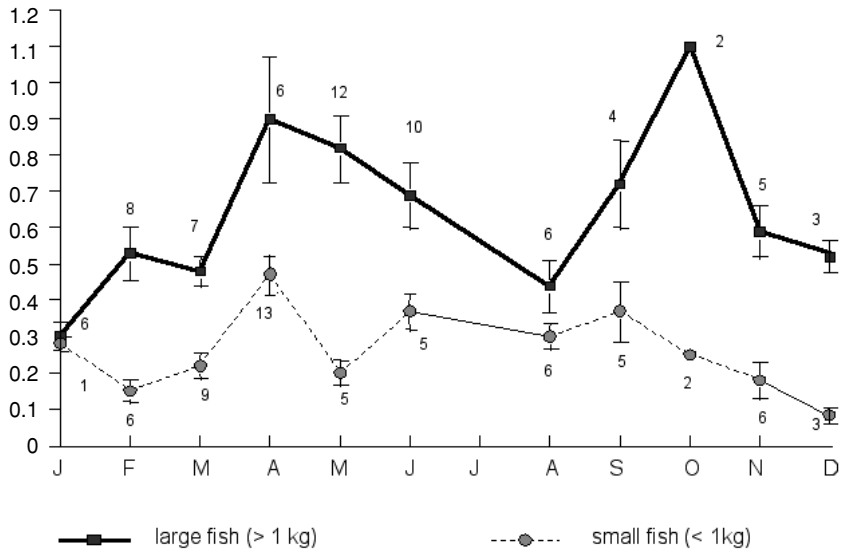

Fig. 2. Seasonal fluctuations in the immunoreactive carp LH (ir-cGtH) content of pituitaries taken from both sexes of large (1-3 kg) or small common carp (0.3-0.99 kg) from commercial harvests of Kibbutz Gan Shmuel (data from Yaron et al., 1984).

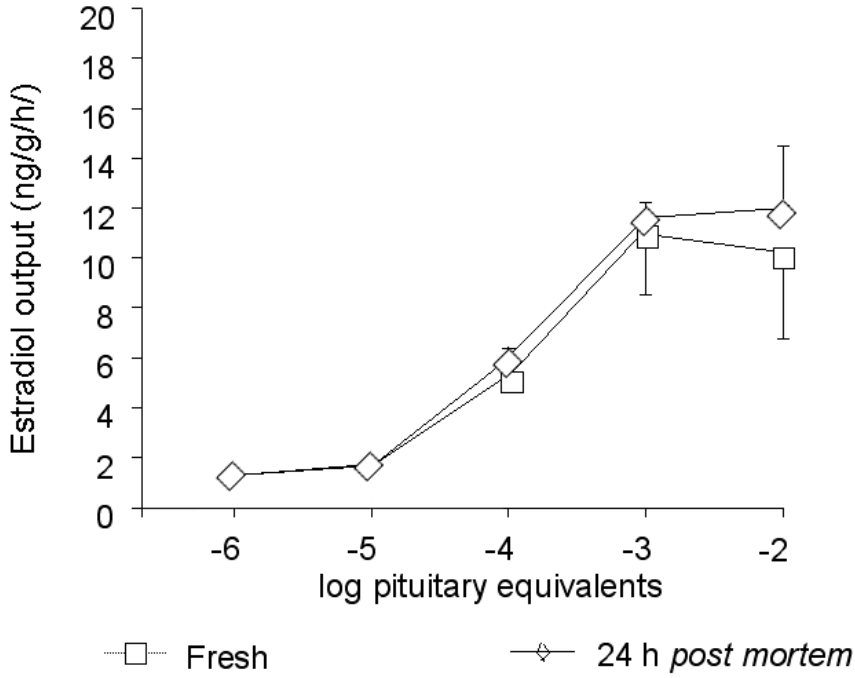

Fig. 3. Biological potency (*in vitro* stimulation of E_2 output from tilapia ovarian fragments) in common carp pituitaries excised from freshly decapitated heads or following storage for 24 h at 4°C (data from Yaron et al., 1984).

(d) *preparation of the extract*. Following removal from the ethanol, glands were hydrated, minced using a tissue grinder (Ultra-Turrax, IKA Werk, Staufen im Breisgau, Germany), and homogenized with a 250-ml Teflon-stainless steel homogenizer. The homogenate was extracted twice overnight at 4°C and, following centrifugation for 60 min at 4000 x *g*, K_2HPO_4 (0.2 M) was added and the extract was heat-treated to inactivate proteolytic enzymes according to Papkoff et al. (1982). The supernatant was cooled and centrifuged again for 90 min at 6000 x *g*.

(e) *extending shelf-life of the extract*. The inert sugar, mannitol, was added as a preservative and aliquots representing 0.5, 1, and 2 pituitaries were placed in ampoules and lyophilized. Sealed ampoules containing the lyophilized extract were stored for 30 days at 4°C or incubated at 37°C to simulate the most adverse storage conditions. Determination of biological activity indicated that storage of the lyophilized extract at 37°C resulted in only partial loss of biological activity (Fig. 5). The lyophilized extract (CCPE) retained most of its gonadotropic activity for more than 4 years when refrigerated at 4°C (Yaron and Kulikovsky, unpublished).

(f) *hormonal profile and progress of oocyte maturation during spawning induction by CCPE*. The hormonal profile of female carp of the Dor 70 line (Wohlfarth et al., 1980), induced to spawn by CCPE, was studied in parallel with the progress of oocyte maturation (Levavi-Zermonsky and Yaron, 1986). To determine the hormone profile, blood was withdrawn from the caudal vessels and assayed for levels of cLH (cGtH), estradiol-17β, and 17α, 20β dihydroxy-4-pregnen-20-one, as in Levavi-Zermonsky and Yaron (1986). To determine the progress of oocyte maturation, biopsies were taken by inserting Tygon tubing (4 mm ID) about 15 cm into the ovary through the genital pore and duct. The excised oocytes were cleared in Serra's fluid [ethanol:formalin

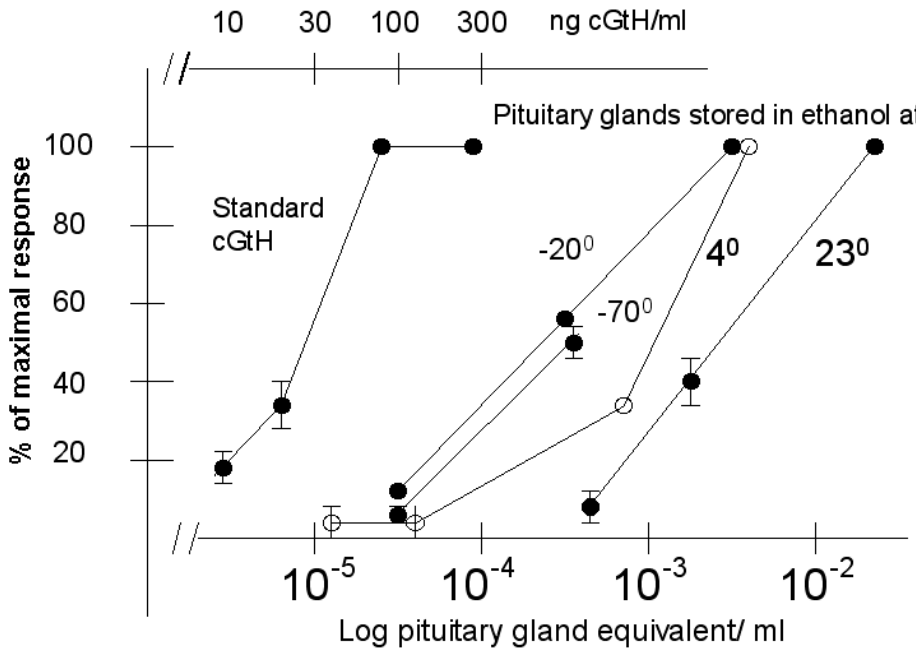

Fig. 4. Harvest and storage of carp pituitary glands. Glands were harvested at the Dag Shan fish processing factory. Some were placed directly into absolute ethanol at room temperature (23°C) and stored for 2 months at 23°C, 4°C, or -20°C. Others were frozen immediately and stored at -70°C for 2 months. Biological potency was compared with standard carp cGtH (Burzawa Gerard, Paris; data from Yaron et al., 1984).

40%:acetic acid (6:3:1)] and the position of the germinal vesicle was determined under a dissecting microscope as in Levavi-Zermonsky and Yaron (1986). Oocyte maturation was classified into the following stages: I - central germinal vesicle (GV), II - migrating GV, III - peripheral GV, IV - GV breakdown (GVBD), V - ovulated eggs in ovarian lumen. Only fish possessing over 60% oocytes at stage II were selected for spawning experiments.

Spawning experiments. A priming dose of CCPE containing 0.07 mg cLH/kg BW was administered at 12:00; the resolving dose containing 0.35 mg cLH/kg BW was given 11 h later. The agent was injected into the dorsal musculature at a volume smaller than 0.5 ml/kg. Following injection, the bottom of each tank was examined frequently for the presence of released eggs, indicating that ovulation had occurred. Females that emitted eggs were anesthetized (ethyl aminobenzoate; 0.15 g/l), blotted, and the eggs were stripped by applying gentle abdominal pressure. Fertilization and incubation were carried out according to Rothbard (1981) and Rothbard and Yaron (1995).

Injection of the priming dose was followed by an increase in the cLH circulatory level to about 80 ng/ml and an increase in the estradiol level to about 8 ng/ml (Fig. 6). By that time most of the oocytes had advanced from stage II to stage III and some had even undergone GVBD. Injection of the resolving dose was followed by a further increase in LH, reaching levels higher than 230 ng/ml. Concomitantly, 17α, 20β-DHP dramatically surged from undetectable levels to 111 ng/ml and estradiol markedly dropped. Four hours following injection of the resolving dose, all oocytes ovulated.

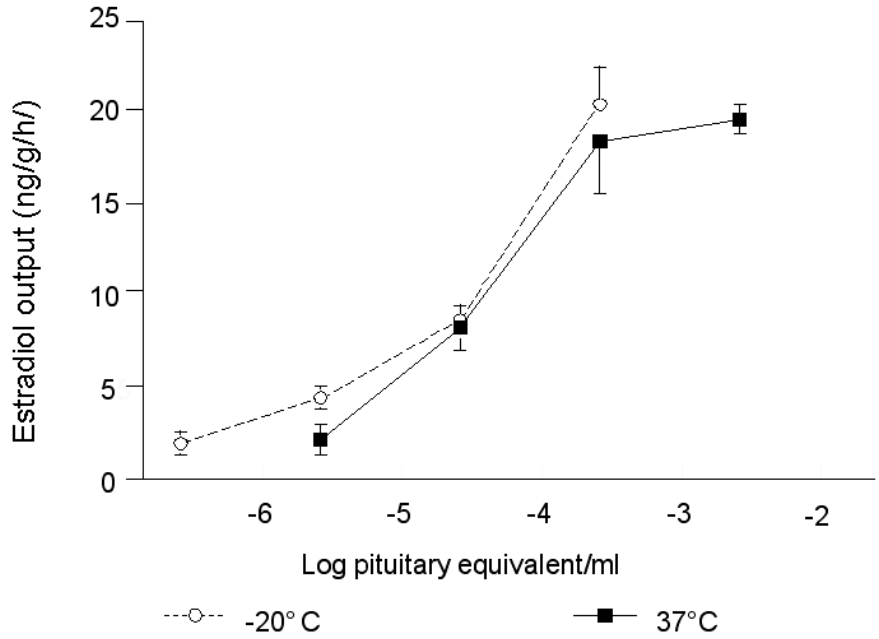

Fig. 5. Long-term temperature resistance of calibrated carp pituitary extract (CCPE), measured as LH biological activity of lyophilized extract stored for 30 days at -20°C or incubated at 37°C, simulating adverse storage conditions (data from Yaron et al., 1984).

In a parallel group injected with a priming dose only, all oocytes reached stage III but did not develop further. It was assumed, therefore, that the moderate increase in the circulating level of cLH following the priming dose synchronizes the maturation progress of all post-vitellogenic oocytes in the ovary (Levavi-Zermonsky and Yaron, 1986). Oocytes remained at stage III until the 17α, 20β-DHP surge that induced the final progress to GVBD and ovulation. It is assumed that the moderate increase in cLH after priming stimulated the formation of MIS receptors on the plasma membrane of the oocytes rendering them sensitive to the MIS surge. Indeed, in sea trout, 6 h of exposure to gonadotropin caused a two to four-fold increase in oocyte and ovarian MIS receptors and the development of oocyte maturational competence (OMC; the ability to complete oocyte maturation *in vitro* in response to exogenous MIS; Thomas et al., 2001).

The decrease in estradiol levels that accompanied the peak in 17β, 20α-DHP demonstrates a shift in the steroidogenic pattern from the formation of estrogens towards the formation of progestogens; this may involve a decrease in 17-20 lyase activity and a rise in 20β hydroxysteroid dehydrogenase activity. In a number of teleost species, including tilapia, two genes encoding 17α hydroxylase (P450c17) have been detected. One is P450c17-I, which is similar to that in tetrapods and also displays the lyase activity that produces C19 steroids (androstenedione or dehydroepiandrosterone) that may serve as precursors for estrogens. This type of P450c17 is expressed in the ovarian granulosa cells of vitellogenic follicles. The other gene, P450c17-II, encodes a 17α hydroxylase that is devoid of lyase activity and fully expressed in oocytes only during final oocyte maturation (Zhou et al., 2007). Irrespective of the mechanism leading to the

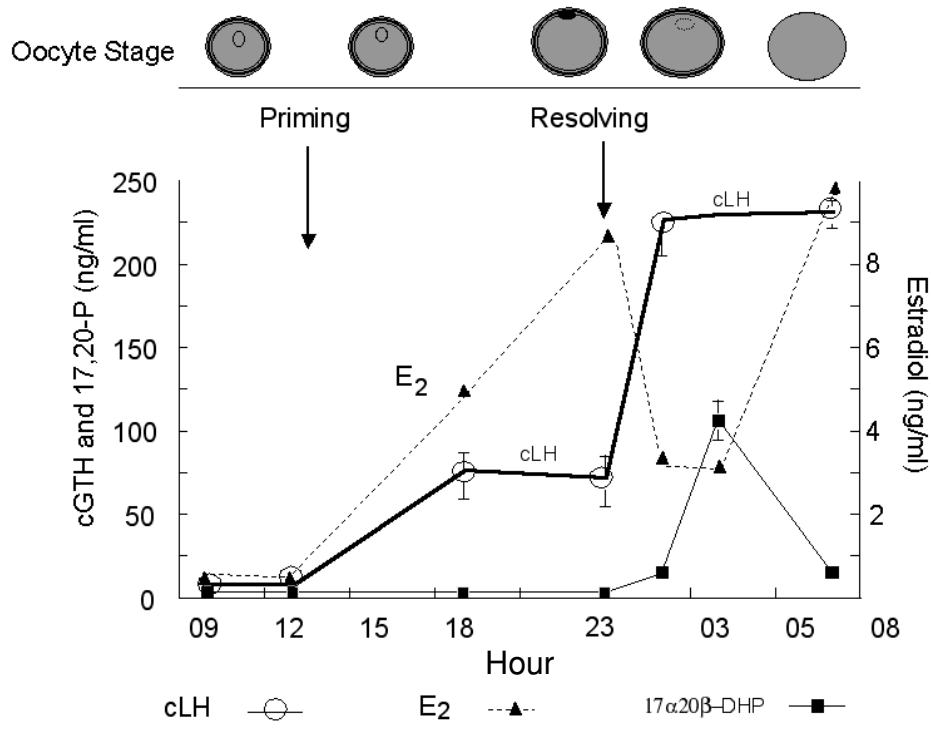

Fig. 6. Progress of oocyte final maturation (illustration at top) and fluctuations in carp LH (cLH), estradiol-17β (E_2), and maturation-inducing steroid (MIS; 17α, 20β-DHP) in the circulation of female carp induced to spawn by CCPE. The priming dose containing 0.07 mg cLH/kg BW was administered at 12:00 (left arrow) and the resolving dose containing 0.35 mg cLH/kg BW was given 11 h later (right arrow). All females ovulated and eggs were stripped the next morning (data from Yaron and Levavi-Zermonsky, 1986).

steroidogenic shift, estradiol and its seven transmembrane receptors (GPR30) in the oocyte maintain meiotic arrest. Therefore, the decrease in estradiol is essential for the resumption of meiosis during oocyte maturation (Pang and Thomas, 2009). Future research is required to determine whether the above mechanism that leads to steroidogenic shift and the role of estradiol and GPR30 also operate in carps.

Selecting suitable females for spawning induction. Vitellogenesis in common carp in Israel is complete by the end of February, when follicle diameter reaches 1 mm. However, at this time of the year not all female carp will ovulate in response to CCPE (Yaron and Levavi-Zermonsky, 1986). Proposed methods for selecting suitable female carp, e.g., abdominal palpation or measurement of maximum body circumference (Rothbard, 1981; Horvath and Tamas, 1984), are subjective and require much skill and experience.

Based on *in vivo* (Brzuska, 1979) and *in vitro* observations (Epler, 1981a,b), two spawning experiments were performed to establish an objective parameter to indicate the suitability of female carp for spawning induction (Yaron and Levavi-Zermonsky, 1986; Yaron et al., 2002a,b). Ovarian biopsies consisting of >40 follicles were taken from Dor 70 carp, cleared in Serra's fluid

as above, and their maturational stage was determined prior to hypophysation with CCPE. Stripping was attempted 7-10 h after injecting the resolving dose.

Results indicated that ovulation was successful only in females in which >66% of the oocytes in their ovarian biopsy had migrating GV (Fig. 7; Yaron and Levavi-Zermonsky, 1986). Attempts to induce spawning in female carp in which more than 34% of the oocytes were still in a central position were unsuccessful. In practice, the biopsy technique is employed by many fish breeders in Israel, especially at the beginning of the breeding season when the suitability of spawners is uncertain.

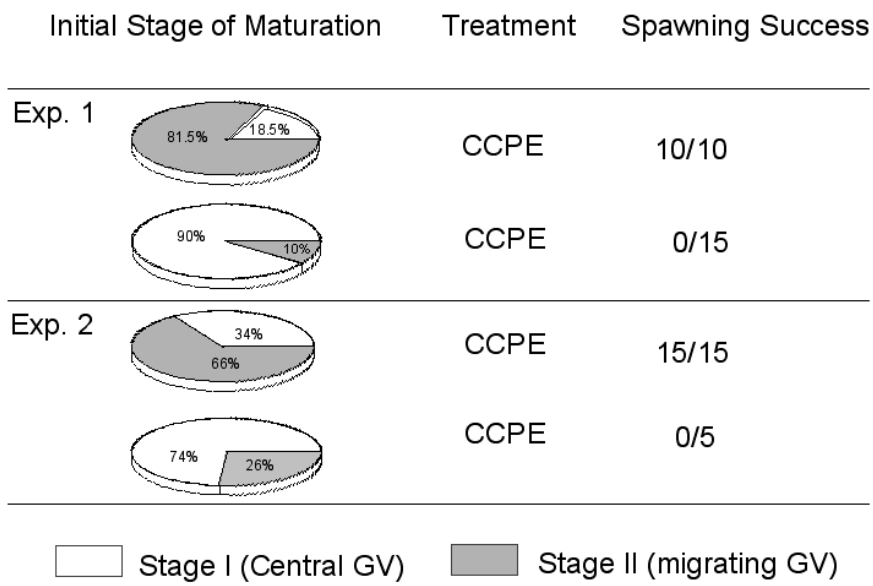

Fig. 7. Results of two experiments to determine the readiness of female common carp for spawning induction. Ovarian biopsies were taken from fish selected for spawning induction by palpation of abdominal softness. Oocytes (>40) were biopsied, cleared, and their maturation stage was determined under a dissecting microscope. Fish were induced to spawn by CCPE as in Fig. 6. Stripping was attempted 7-10 h following administration of the resolving dose. No response, or partial or abnormal release of eggs, was regarded as unsuccessful. Before treatment, two main cohorts of post-vitellogenic oocytes were distinguished: stage I oocytes with central germinal vesicle (GV) and stage II oocytes with migrating GV. The percent of initial oocyte stages was compiled *a posteriori* from all females in the cohort (modified from Yaron et al., 2002a,b).

The Hypothalamic Approach

During the early 1990s, the production of ornamental cyprinids (koi and goldfish) in Israeli aquaculture increased while the amount of common carp processed by the industry decreased. Therefore, the supply of pituitaries could no longer meet the CCPE demand. This situation, together with the risk of spreading pathogens from pituitary glands of donor fish, motivated the introduction of the hypothalamic approach into local aquaculture. The hypothalamic approach is based on stimulation of endogenous GtH release by a superactive analogue of gonadotropin-releasing hormone (sGnRHa).

Administration of GnRHa is very effective if given in slow-releasing devices made with cholesterol pellets (Crim et al., 1988a) or biodegradable polymers (Zohar, 1988; Zohar et al., 1994; Mylonas et al., 1995; Barbaro et al., 1997). Slow-release systems are particularly effective in fish with an asynchronous mode of ovulation such as gilthead sea bream or striped bass. In synchronous spawners such as common carp there is no need for sustained release of LH as a single surge is necessary to induce ovulation. Nevertheless, as LH release in cyprinids is also controlled by a strong dopaminergic inhibition (Chang and Peter, 1983; Chang et al., 1984; Peter et al., 1986), the GnRH effect can be augmented by the addition of a dopamine receptor antagonist. The use of such a combination is known as the Linpe Method, named after Professor Hao R. Lin and the late Professor R.E. Peter. The Linpe Method has been successfully employed in many cyprinids (reviewed by Lin et al., 1986; Peter et al., 1988, 1993; Yaron, 1995).

To adapt the Linpe Method to conditions in Israeli aquaculture, a project was initiated with the following objectives: (a) to calibrate doses of a GnRH analogue and a dopamine antagonist that will induce spawning in common carp when given in a single injection, (b) to determine whether the ovulatory response varies when fish are treated at different times of the day, (c) to record the progress of oocyte maturation in accordance with circulating levels of cLH, estradiol-17 β, and 17α, 20 β-DHP, (d) to determine the precise latency between treatment and ovulation as a function of temperature, and (e) to compare these parameters with those in fish induced to spawn by CCPE treatment.

GnRH and dopamine antagonist dosages. For sGnRHa ([D-Arg^6, Pro^9-Net]-sGnRH), 10 µg/kg BW was the minimal effective dose for spawning induction in carp when given together with the dopamine receptor antagonist metoclopramide (MET; Drori et al., 1994). Although this superactive analogue was no more potent than the mammalian superactive analogue ([D-Ala^6, Pro^9-NEt]-LHRH) in rainbow trout (Crim et al., 1988b; Weil et al., 1992), winter flounder (Crim et al., 1988b), and gilthead sea bream (Zohar, 1989; Zohar et al., 1990), it was superior in elevating circulating GtH and induction of ovulation in the carp (Lin et al., 1988). In spite of its inferior potency in goldfish compared to domperidone or pimozide (Omeljaniuk et al., 1987), metoclopramide was selected because of its aqueous solubility and ease of use by the farmer. The lowest effective dose of MET was 20 mg/kg. Even the exaggerated dose of 100 mg/kg did not result in any deleterious effect in carp, except for a temporary darkening of the skin around the injection site which disappeared within a few hours (Drori and Levavi-Sivan, unpubl.). The combination of 10 mg/kg sGnRHa and 20 mg/kg MET was named Dagin (Fig. 8).

Is there a daily rhythm in fish sensitivity to spawning induction? According to studies in *Sparus aurata* (Zohar, 1988), *Dicentrarchus labrax* (Alvarino et al., 1992), and *Cyprinus carpio* in Poland (Bieniarz et al., 1985), the success of spawning induction depends on the time of day at which treatment is given. In contrast, our study demonstrated a similar latency and ovulatory response to Dagin irrespective of the time of treatment. Further, the increase in cLH following Dagin treatment was similar in carp treated in the morning and in the night (Drori et al., 1994).

Hormonal profile and oocyte maturation. The rise in circulating cLH following a single dose of Dagin was very gradual and reached a peak after 14 h, when ovulation had already started (Fig. 9). In contrast, cLH rose sharply after the resolving dose in fish injected with CCPE, reached a peak 4 h later, and started to decline even before ovulation occurred. Estradiol increased in response to both the priming dose of CCPE and to Dagin injection and declined prior to ovulation in the Dagin treated fish. The surge in 17α, 20β-DHP was similar in both treatments and occurred 2-4 h before ovulation. It seems that the very gradual increase in cLH in response to the single injection of Dagin replicated the kinetics of cLH following the two injections of CCPE. This may explain why treatment with Dagin does not require priming and is effective even if given in a single injection, which reduces labor and avoids unnecessary handling stress to the broodstock.

Latency of response. In agreement with results obtained in other cyprinids (Horvath, 1978; Zonneveld, 1984; Kucharczyk et al., 1997), the latency of response was inversely related to

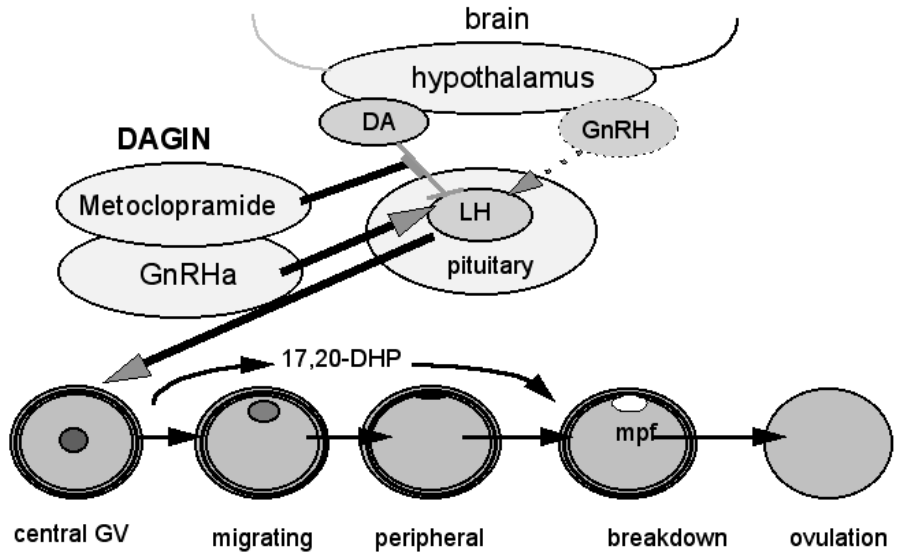

Fig. 8. Proposed mechanism of action of Dagin (10 μg/kg GnRHa + 20 mg/kg metoclopramide), based on the Linpe Method. Injected GnRHa stimulates the secretion of LH from the pituitary, while metoclopramide reduces the inhibitory effect of dopamine on the release. In turn, LH augments secretion of the maturation-inducing steroid (MIS; 17α, 20β-DHP), followed by formation of the maturation-promoting factor (MPF) in the oocyte, which is involved in resumption of meiosis and manifested by germinal vesicle breakdown and ovulation.

water temperature (Fig. 10). Latency was constant in response to both CCPE and Dagin at 22.5-24°C, considered the optimal temperature range for spawning in common carp (Horvath, 1978). The longer latency in Dagin-treated fish is probably due to the fact that two sequential processes take place after injection with Dagin (stimulation of LH release and the response of the ovarian follicle to LH), rather than the single process (ovarian response to exogenous LH) that occurs after injection with CCPE.

Latency variance, spawning success, survival of embryos. Seven commercial spawning operations were conducted in the Gan Shmuel Fish Breeding Center (Kulikovsky et al., 1996). On the same day, female carp (1.5-3.5 kg; koi and Dor 70 variants; 10-33 females per operation) were randomly chosen for spawning using either CCPE or Dagin. The spawning ratio was similar in both treatments. Embryo viability was also similar in both treatments, except in one case at the end of August when embryo viability in fish treated with CCPE was 20% better than in fish treated with Dagin. Latency to ovulation was considerably greater in the Dagin treatment than with CCPE (up to 6 h difference) and variability in latency was significantly greater (Fig. 11). Presumably, the greater variability in latency in Dagin-treated carp is due to the longer chain of physiological events compared to that in CCPE-treated carp.

Dagin treatment during February (early season) or August (late in the season) occasionally fails to induce ovulation and spawning (Yaron and Kulikovsky, unpubl.). Such failure may be attributed to the low LH content in the pituitaries during these months (Fig. 2). Although GnRH reportedly stimulates synthesis of LH in the carp pituitary (Kandel-Kfir et al., 2002), protocols designed to increase the LH level in fish with deficient pituitaries require further research.

Dagin in other carp strains and fish. In addition to koi and Dor 70, Dagin is effective in other common carp, depending on the strain. The response to Dagin varies according to the source of

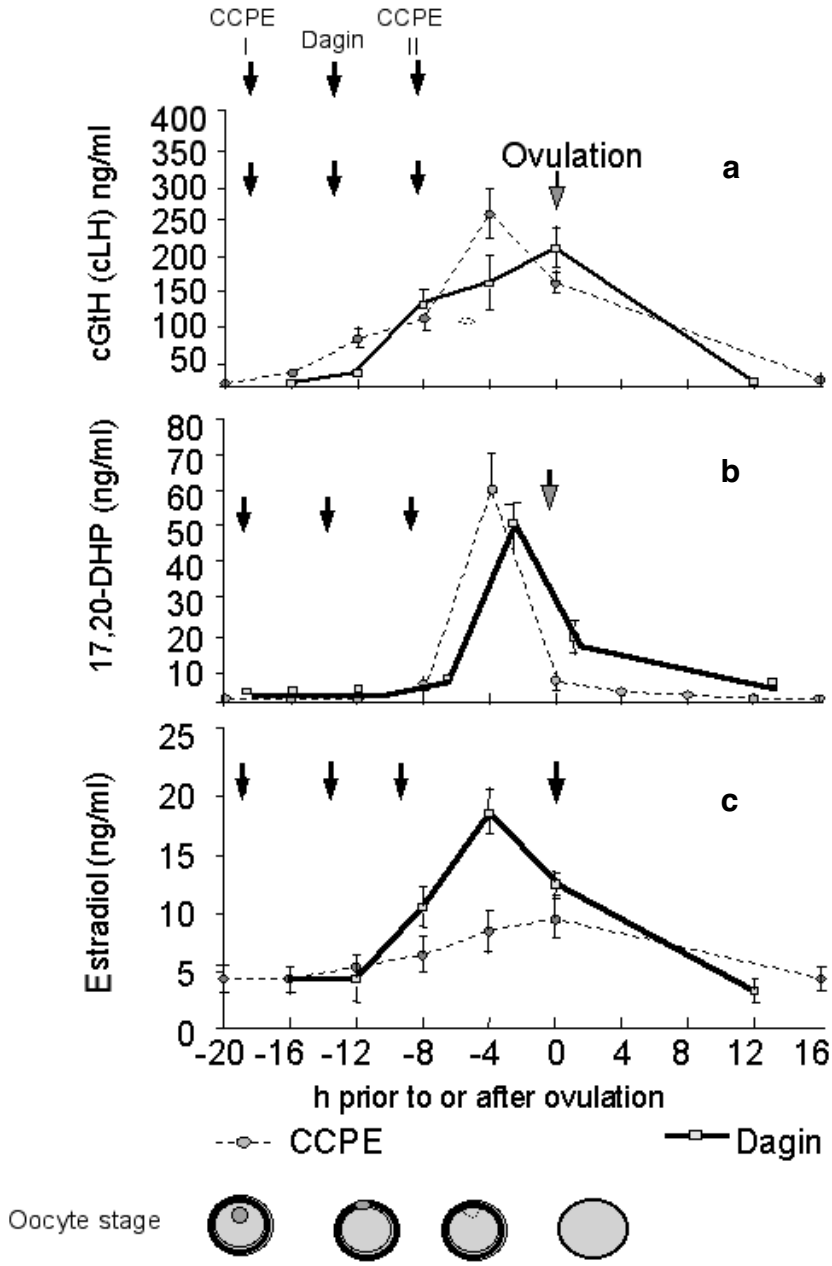

Fig. 9. Circulating levels of (a) cGtH (cLH), (b) 17α, 20β-DHP, and (c) estradiol in female common carp (Dor 70 line, 1-1.5 kg BW) after injection of CCPE (priming and resolving doses as per Fig. 6) or Dagin (as per Fig. 8). Blood was sampled at intervals to measure hormone levels and ovarian biopsies were taken to determine the progress of oocyte maturation. Hormone levels are expressed as means ±SEM (data from Drori et al., 1994). Bottom panel shows progress of oocyte maturation during spawning induction (from left): migrating GV (stage II), peripheral GV (stage III), GV breakdown (stage IV), and ovulated egg (stage V).

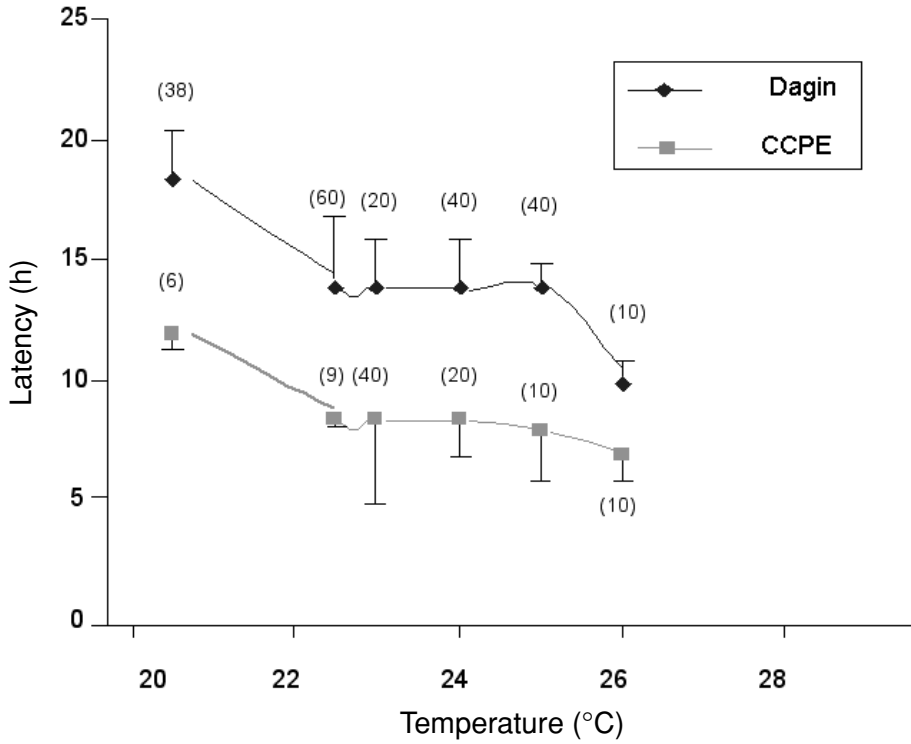

Fig. 10. Temperature determines latency (time between injection of Dagin or resolving dose of CCPE and ovulation, determined by the initial appearance of released eggs in the tank). Bars represent the maximum or minimum latency range at each temperature. Number of fish in parentheses (data from Drori et al., 1994).

the fish and its genetic line (Brzuska, 2005, 2006). The application of Dagin to common carp of the Hungarian strain induced twice the rate of spawning as that in female strain 6 (Polish line). Although a single dose of Dagin was less effective than two injections of Ovopel (Linpe Method) or crude pituitary extract in raising the percentage of ovulating females and weight of ovulated eggs, Dagin did not increase the number of deformed larvae in comparison with the number obtained following the other treatments (Brzuska, 2005). Experiments under local conditions (environment and genetic source of fish) should be carried out before transferring technology protocols from one country to the other. Nevertheless, Dagin was effective for spawning in other cyprinids, such as various lines of goldfish (Rothbard et al., 1997a), black carp (Rothbard et al., 1997b), grass carp (Rothbard et al., 2000), the African cyprinid, *Labeo victorianus* (Rutaisire and Booth, 2004), and also the northern pike, *Esox lucius* (Tams, 2003).

Recombinant Gonadotropins for Spawning Induction

In spite of its efficiency as a spawning-induction agent, Dagin has disadvantages compared to pituitary extract. Its latency is relatively long and variable, and it is less effective early and late in the season when the pituitary LH content is low. Thus, we began to search for a better prepara-

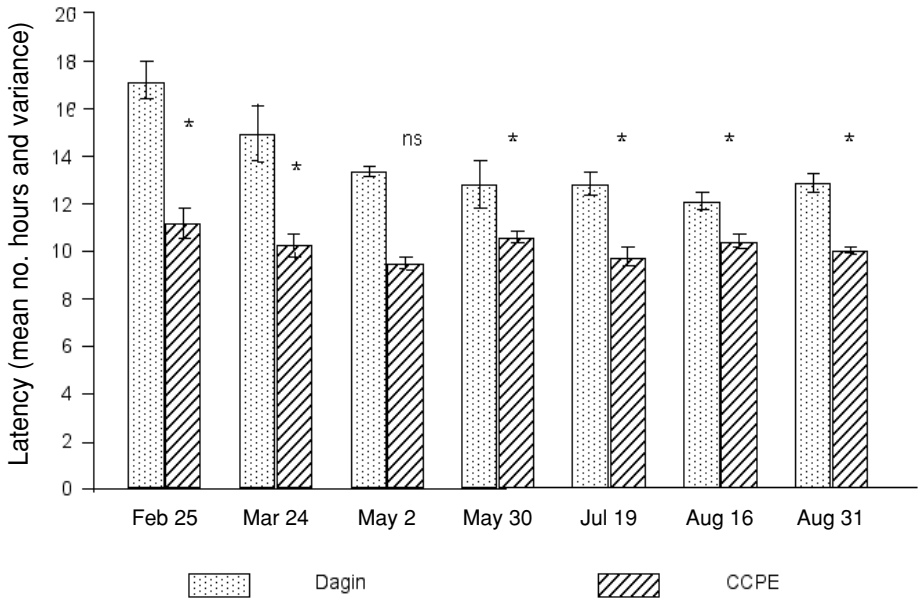

Fig. 11. Comparison of latency (± variance) in seven spawning operations of common carp (koi and Dor 70 lines) in a commercial hatchery (Fish Breeding Center, Gan Shmuel, Israel). On each date, fish were injected with either CCPE or Dagin, as per Figs. 6 and 8. Asterisks denote significant differences ($p<0.05$) in variance between treatments (from Kulikovsky et al., 1996).

tion, taking advantage of molecular technology. Using molecular tools, we succeeded to produce a recombinant LH of tilapia (tLH) that includes both β and α subunits.

We chose to express the recombinant gonadotropins of tilapia in the methylotrophic yeast, *Pichia pastoris*, which has several advantages over other known expression systems: (a) high-level production of recombinant proteins, (b) relatively inexpensive, and (3) capacity to carry out post-translational modifications (e.g., glycosylation) resembling those of vertebrate cells.

Mature protein-coding sequences of tilapia LHβ and LHα were joined to form a fusion gene that encodes a "tethered" polypeptide in which the tLH β -chain forms the N-terminal part and the α-chain forms the C-terminal part. A "linker" sequence of six amino acids (three Gly-Ser pairs) was placed between the β and α-chains to assist in chimerization of the subunits, and a six-His tail was placed at the end of the β-subunit to enable purification of the recombinant protein (Kasuto et al., 2005). Two recombinant plasmids containing either tLH β or tilapia glycoprotein hormone α (tGPα) subunits were constructed (products A and B, Fig. 12, respectively) to form fusion genes to be subcloned into the *Pichia* expression vector. The oligonucleotide primers used to clone the subunit DNAs from the pituitary of *Oreochromis niloticus* were designed according to nucleotide sequences of *O. mossambicus* tGPα (Gur et al., 2001) and tLH α (Rosenfeld et al., 1997). Constructs were used to transform *P. pastor*is strain GS115 by electroporation, resulting in insertion of the construct in the alcohol (methanol) oxidase gene (AOX1) locus of the yeast. Transformants were selected for high copy number according to their ability to grow on agar containing the antibiotic G418 at various concentrations. The protein was

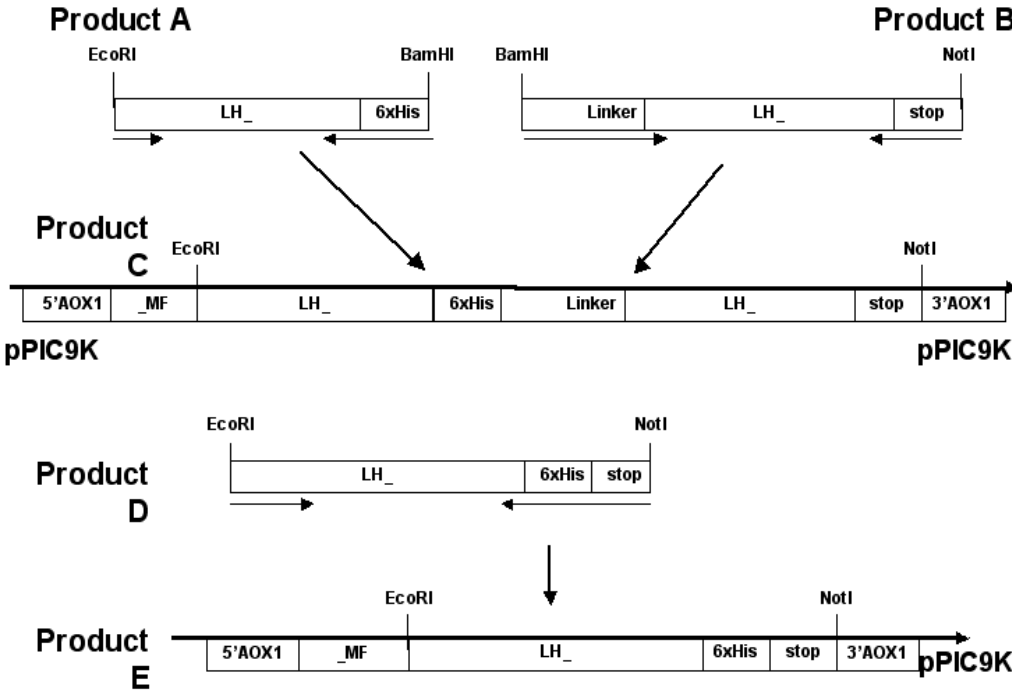

Fig. 12. Construction of the expression vector for tilapia LHβα^{His}. The expression vector pPIC9K is an *Escherichia coli-Pichia pastoris* shuttle vector with sequences required for selection in each host. It has 5' promoter and 3' transcription-termination sequences of the alcohol (methanol) oxidase gene (AOX1) flanking the cloning site into which tilapia LHβα was introduced. The vector has a yeast mating factor (αMF) signal peptide downstream of the AOX1 promoter to which tilapia LHβα was fused (from Kasuto et al., 2005).

expressed in a shaker flask and harvested 72 h after induction by methanol. The recombinant gonadotropin (tLH βα) was purified using one-step nickel batch purification (Fig. 13).

Western blot analysis of the recombinant tLH βα resolved by SDS-PAGE showed its mobility to be similar to that of standard tLH purified from the pituitary. Moreover, the recombinant tLH βα was effective in stimulating secretion of 11-ketotestosterone from mature testes (Kasuto et al., 2005), estradiol from vitellogenic follicles (Levavi-Sivan et al., 2008), and DHP from post-vitellogenic ovaries of carp (Aizen et al., 2008). It is anticipated that the same technology can be applied to other fish in aquaculture.

Conclusion

In conclusion, intensified culture of edible and ornamental cyprinid fish in Israel has required the optimization of spawning techniques. This has been achieved through collaboration between scientists, commercial hatcheries, and the fish-processing industry. Fish breeders in Israel can now choose between two spawning agents, the calibrated and pre-tested carp pituitary extract (CCPE) or Dagin, according to their needs. It is anticipated that the next generation of spawning induction agents will utilize molecular tools to produce recombinant gonadotropin at an affordable cost.

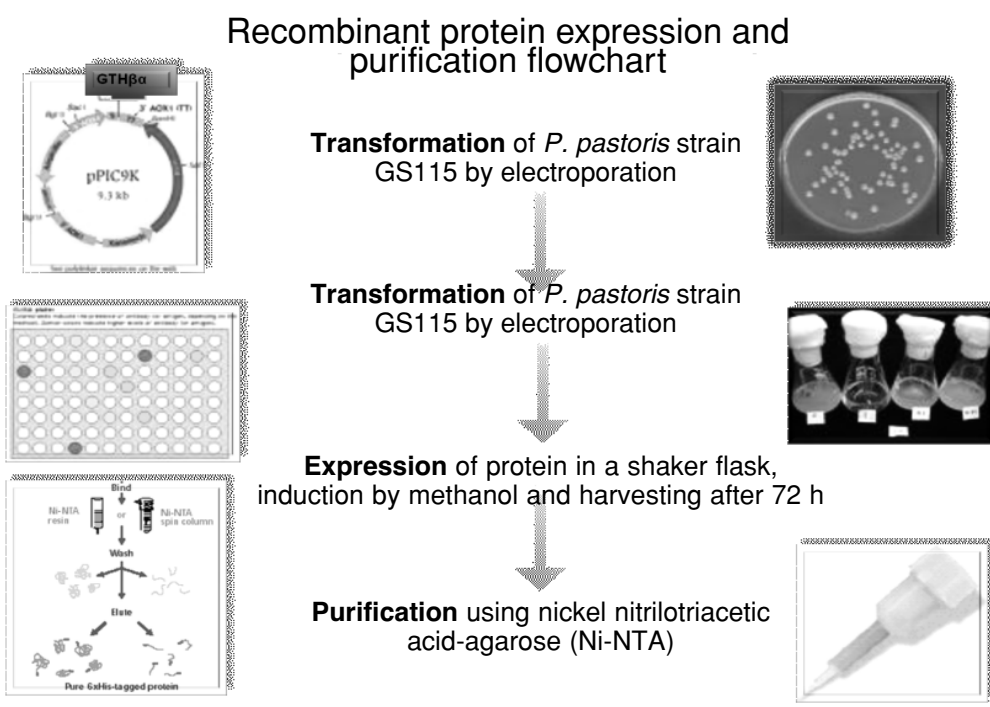

Fig. 13. Preparation of recombinant tilapia LH in the yeast *Pichia pastoris*.

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