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A Study of Annual Female Reproductive Cycle: the Ovarian Steroid Profile in the Cyprinids, *Barilius vagra* and *Cyprinion watsoni* (Teleostei)

By

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By:
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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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2002

In the Glorious name of Allah, the most Compassionate,
the most Merciful, the most Beneficent and the
Master of the Day of Judgment

Dedicated to
My parents and my respected Professor Dr. M.A. Hafeez

CERTIFICATE

This thesis, submitted by Miss Amina Zuberi, is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, as satisfying the requirements for the degree of Doctor of Philosophy in Biological Sciences.

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ABSTRACT

The present work on two freshwater teleosts, *Cyprinion watsoni* and *Barilius vagra* (Cyprinidae, Teleostei) was conducted to study (1) monthly steroid profile in relation to seasonal changes in ovarian development, (2) relative importance of various ovarian steroids in inducing maturation of oocytes *in vitro* as well as the role of human chorionic gonadotropin (hCG) in this process, and (3) metabolic potential of the ovary of *Cyprinion watsoni in vitro* in the presence of selected steroid substrates, given singly or jointly with hCG.

The study shows that both *C. watsoni* and *B. vagra* are asynchronous spawners with a breeding season that lasts from May to August. The histological picture of the ovaries of these fish matched the seasonal GSI. The mean GSI in the two species was low in the postspawning months, reached a peak in April and gradually declined during the spawning season (May to August) to its lowest values in the postspawning months of September to December. Following initial vitellogenic progress that began in January/February and reached its peak in April, the vitellogenic and maturing oocytes coexisted during the active spawning months.

The determination of ovarian steroids *in vivo* and *in vitro* was done by solid phase extraction of steroids from ovarian samples and the incubation medium, using Sep-Pak C18 and resolution by reverse phase gradient mobile HPLC at 254 and 280 nm. The steroids identified *in vivo* during the year were estriol (E: estra-1,3,5 (10)-triene-3-16α, 17β-triol), estrone (E_1: estra-1,3,5 (10)-triene-3-ol-17-one), estradiol 17β (E_2 : estra-1,3,5 (10)- triene-3, 17β- diol), testosterone (T: 17β- hydroxy- 4- androsten- 3- one), androstenedione (AD: 4-androstene-3, 17-dione), 11β-hydroxyandrostenedione (11B-OHA: 11β-hydroxy-4-androstene-3, 17-dione), 19-hydroxyandrostenedione (19-OHA: 19β-hydroxy-4-androstene-3, 17-dione), progesterone (P_4: pregn-4-ene-3, 20-dione), 17-α-hydroxyprogesterone (17-OHP: 17α-hydroxypregn-4-ene-3, 20-dione), 17α-20β-dihydroxypregn-4-ene-3-dione (17,20βP: 17, 20β-dihydroxypregn-4-ene-3-one), 11-

deoxycorticosterone (DOC: 21-hydroxypregn-4-ene-3,20-dione), corticosterone (B: 11β, 21-dihydroxypregn-4-ene-3, 20-dione), cortisol (F: 11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione) and aldosterone (ALDO: 11β, 21-dihydroxypregn-4-ene-3, 20-dion-18-al). The levels of the three estrogens increased in parallel with the initial vitellogenic progress during January to April, with peak levels in March/April followed by only gradual decline in the spawning season (May to August). E_1 appeared as a prominent estrogen in both species and matched E_2 both in level and persistence at appreciably high levels during the spawning season. The seasonal profile of T and AD was also similar to that of estrogens, suggesting a role in vitellogenesis as well as maturation of the oocytes. 11β-OHA and 19-OHA waxed and waned during the year and were the dominant androgens in both species. The concentration of P_4 was generally low during the year in both species. 17-OHP and 17, 20βP showed peak concentration coincident with vitellogenic peaks and onset of the spawning season and prevailed at appreciably high levels during major part of the spawning season (period of oocyte maturation/ovulation and oviposition). The presence of DOC, B and F throughout the year in the two species demonstrated that their ovaries are capable of synthesizing these steroids. In both species, DOC and B appeared as dominant corticoids. Peak levels of DOC and B in *C. watsoni* matched the early to late recrudescent phase; while in *B. vagra* high levels of these steroids occurred in the periovulatory and spawning season. The levels of F were generally and relatively low throughout the year in both species. Species differences were evident in that its concentration was slightly higher in *B. vagra*, whereas in *C. watsoni* it persisted for a longer duration. The detection of ALDO was not surprising since it has also been identified in a few other species. The *in vivo* profile collectively showed a pattern that matched the asynchronous ovarian development and affirms the view that the estrogens principally support vitellogenic growth and the progestins oocyte maturation. The androgens appear to be associated with both vitellogenesis and maturational progress. There is need to experimentally determine the precise physiological significance of 11-oxoandrogens and the corticoids identified presently in the two species. It is further noted that a more precise definition of the association between the ovarian steroids and the ovarian stages may be achieved by analysis at shorter sampling intervals than has been possible in this study.

The results of the *in vitro* work on oocyte maturation reveals that 17, 20βP is the most potent MIS in the two species. While the estrogens tested *in vitro* were entirely ineffective in causing significant GVBD, both the androgens and progestins caused significant GVBD, the response being both time and dose-dependent. However, only 17, 20βP caused the earliest GVBD and with near physiological dosage (0.01 ug/ml, 24 hr incubation, *C. watsoni*). In *B. vagra*, even DOC had a significant GVBD effect but again 17, 20βP turned out to be the most potent MIS. Human chorionic gonadotropin (hCG) also caused slight but significant GVBD in both species and strongly promoted the GVBD response to all exogenous steroids, especially the progestins.

Treatment of the ovarian follicles of *C. watsoni* with hCG alone or jointly with the various steroid substrates and recovery of metabolites in the incubation medium demonstrated existence of a repertoire of enzyme pathways in the fully grown ovarian follicles. Whereas the *in vitro* and *in vivo* analyses support existence of at least cytochrome P450aromatase, 17-hydroxysteroid dehydrogenase (17-HSD), 20β-hydroxysteroid dehydrogenase (20β-HSD), 11-hydroxylase, 16-hydoxylase, aldosterone synthetase (P450aldo) and sulphuronyl and glucuronyl transferases, the presence of a number of unknown peaks in the chromatograms suggests existence of other enzymes as well. Further implication of this latter observation is that while 17, 20βP appears to be the most potent MIS *in vivo* and *in vitro* in the two species, definitive conclusion in this regard must be deferred until further work has been done to identify these as yet unknown steroids, some of which may be triols or tetrols.

The presence of conjugated estrogens, androgens and progestins both *in vivo* and *in vitro* not only reflects the ability of the ovaries to intramurally deactivate and excrete these steroids but also invites attention for future investigations to determine whether the conjugated steroids have other biologically important functions, pheromonal or other, in fish reproduction.

The present study was undertaken against the background of total neglect in this country of a very fundamental area of reproductive physiology of fishes. It thus represents a "ground-breaking" effort to overcome technical handicaps and paves the way for further in-depth work on these two species and other local commercially important species.

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List of Abbreviations

11B-OHA	11β-hydroxyandrostenedione
11-DOC	11-deoxycortisol
11-KT	11-ketotestosterone
11-oxygenated androgens	11β-hydroxyandrostenedione and 11- ketoandrostenedione
11β-OHT	11β-hydroxy testosterone
17,20βP	17, 20β-dihydroxypregn-4-ene-3-one
17-HSD	17-hydroxysteroid dehydrogenase
17-OHP	17α-hydroxyprogesterone
19-OHA	19-hydroxyandrostenedione
20β-HSD	20β-hydroxysteroid dehydrogenase
20βS	20β-dihydroxyprogesterone
AD	androstenedione
ALDO	aldosterone
Anova	analysis of variance
B	corticosterone
BSS	Basic Salt Solution
CGV	central germinal vesicle
DMRT	Duncan's Multiple Range Test
DOC	11-deoxycorticosterone
E	estriol
E_1	estrone
E_2	17β- estradiol

EA	ethyl acetate
F	cortisol
G	glucuronide
Gluco. transferase	glucuronide transferase
GSI	gonadosomatic index
GV	germinal vesicle
GVBD	germinal vesicle break down
hCG	human chorionic gonadotropin
HPLC	high pressure liquid chromatography
MIS	maturation inducing steroid
P_4	progesterone
$P450_{scc\ 2}$	cytochrome P450 side chain cleavage-2
$P450_{aldo}$	aldosterone synthetase
$P450_{arom}$	P450aromatase
$P450_{c\text{-}11}$	cytochrome P450 11-hydroxylase
$P450_{C17}$	cytochrome P450 17-hydroxylase / 17, 20-lyase
P450c21	cytochrome P450 side chain cleavage (c21)
S.E	standard error
SO4	sulphate
T	testosterone
TFA	trifluoroacetic acid
UKN	unknown i.e. metabolite not co-eluted with reference steroid

INTRODUCTION

Reproductive cycles of fishes and the underlying physiological mechanisms have been of profound interest to fish biologists the world over. An understanding of these is not only crucial from a comparative point of view but also for developing methods for controlling reproduction of economically important species of teleosts. In Pakistan, much of the focus of research in this field has been confined to descriptions of seasonal reproductive cycles. Instances are rare where environmental and hormonal factors that govern reproductive seasonality in local species of fish have been analyzed *in vivo* (Shaikh and Hafeez, 1993; Shaikh *et al.*, 1991) or *in vitro* (Haider, 1985; Ghaffar, 1988; Zuberi, 1990). The *in vitro* studies by these latter workers have been largely of a preliminary nature. In fishes, as in other vertebrates, the gonads respond to environmentally mediated hypothalamo-hypophyseal influences and sequentially pass through stages of gametogenesis and maturation of the gametes, culminating in spawning episodes. Gonadal steroids that also constitute the focus of the present investigation regulate these sequential events. A voluminous body of information currently exists on steroid profiles, steroid biosynthesis and the temporal relationship of the steroids with breeding cycles of fishes in general and teleosts in particular. Several comprehensive reviews of the work done on both testicular and ovarian steroids during the last several decades are available (Idler, 1972; Colombo and Belvedere, 1977, Colombo *et al.*,1978a; Kime, 1991, 1993; Goetz, 1983; Fostier *et al.*, 1983, 1987; Scott and Canario, 1987; Scott *et al.*, 1998; Nagahama, 1994a; Nagahama *et al.*, 1982, 1994; Borg, 1994). The present work reported herein was undertaken to examine the seasonal ovarian steroid profile of two local freshwater cyprinids, *Cyprinion watsoni* and *Barilius vagra* with a view to relating it to their breeding seasonality. Also included in this study is analysis of steroids that may mediate maturation of the oocytes *in vitro* and *in vivo*.

Progressive development of ovarian follicles involves several steps (reviews: Goetz, 1983; Selman and Wallace, 1986; Nagahama, 1983; 1994a; Nagahama *et al.*, 1994). Initially, the oocyte passes through periods of primary and secondary growth during which its surrounding envelopes of thecal cells, granulosa cells and chorion are added, sequestration of hepatically synthesized vitellogenin results in marked increase in the size of the follicles. Until this time, the oocyte remains arrested in the first meiotic prophase. The postvitellogenic oocyte now enters maturation phase during which reinitiation of meiotic events, migration of the germinal vesicle (GV) to the oocyte periphery and its ultimate breakdown (GVBD) occurs signaling completion of the meiotic prophase-1. The oocyte progresses towards metaphase-2, increases further in size by hydration and becomes a fertilizable egg. It is ovulated and the fish is ready for spawning, environmental conditions permitting.

These developmental events in the ovaries of teleosts are regulated by steroids that are synthesized therein under the direct influence of two gonadotropins, GTH-I and GTH-II (Ng and Idler, 1978; Peter, 1981; Idler and Ng, 1983; Suzuki *et al.*, 1988; Swanson, 1991; Schulz, 1995; Hassin *et al.*, 1995; Mylonas *et al.*, 1997). Updates of the earlier studies on gonadal steroids (reviews: Idler, 1972; Kime, 1979, 1980; Fostier *et al.*, 1983; Goetz, 1983) by Kime (1993), Nagahama *et al.* (1994), Borg (1994), Scott *et al.* (1998) show that a wide range of estrogens, androgens and progestogens, both "classical" and "non-classical", are synthesized in the ovaries of fishes. According to the classical view, estradiol 17β regulates the synthesis of hepatic yolk that is then sequestered into the oocyte. GTH-I has been shown to activate the enzyme P450 aromatase that converts testosterone to estradiol 17β and androsteronedione to estrone; the entire process occurring, respectively, in the thecal cells and granulosa cells of the follicle (two-cell salmonid model: Kagawa *et al.*, 1982a, b, 1985; Young *et al.*, 1984; Scott and Canario, 1987; Kime, 1993; Nagahama *et al.*, 1994) or entirely in the granulosa cells (non-salmonid model: Wallace and Selman, 1986; Petrino *et al.*, 1989; Kime

1993). On completion of vitellogenesis, GTH-II stimulates synthesis of maturation inducing hormone (MIH/MIS) via 20β-hydroxytsteroid dehydrogenase (20β-HSD), which in most species appears to be 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20βP) (Scott *et al.*, 1982; Scott and Canario; 1987, 1990, 1992; Scott and Turner, 1991; Kime, 1993; Nagahama *et al.*,1994). This sequence of steroid biosynthesis and regulation of oocyte development in fish ovary principally based on work on salmonids has been subjected to critical analysis by examination of several other major groups of teleosts, revealing great variety in steroid biosynthetic pathways and seasonal correlations (Horvath 1978, Kime, 1979, 1990, 1992; Campbell *et al.*, 1980; Bohemen, 1980; Godovich *et al.*, 1982; Bromage *et al.*, 1982; Kagawa *et al.*, 1983a, b, 1984; Scott *et al.*, 1980, 1983; Scott and Canario, 1987; Scott and Sumpter,1989; Scott and Turner, 1991; Scott and Canario, 1990; Scott and Baynes, 1982; Scott *et al.*, 1982, 1998; Kime and Haider, 1983; Fitzpatrick *et al.*, 1986; Yaron and Levavi-Zermonsky, 1986; Manning and Kime, 1984; Petrino *et al.*, 1989; Rinchard *et al.*, 1993; King *et al.*, 1994a, b; Malison *et al.*, 1994, 1997; Mananos *et al* 1997; Asturiano *et al.*, 2000; Susca *et al.*, 2001). In view of the diversity of the teleosts and the reproductive strategies adopted by individual species models based on a single group of teleosts can hardly be considered of universal application. This constituted one of the additional considerations that compelled the present work on the selected cyprinid species in an attempt to contribute to the wealth of information already available on the seasonality and biosynthesis of ovarian steroids.

In vitro bioassays have been of universal value in evaluating the relative effectiveness of gonadal steroids in inducing final oocyte maturation, hormonal regulation and ovulation in a variety of teleosts (Idler *et al.*, 1960; Schmidt and Idler, 1962; Fostier *et al.*, 1973; Jalabert and Breton, 1973; Jalabert, 1976; Sundararaj and Goswami, 1977; Goetz and Bergman, 1978; Iwamatsu, 1974, 1978, 1980; Duffey and Goetz, 1980; Campbell *et al.*, 1980; Nagahama *et al.*, 1980, 1983, 1994; Sower and Schreck, 1982; Scott and Baynes, 1982; Young *et al.*,

1982, 1983a, b, 1986; Yamauchi *et al.*, 1983; Nagahama and Adachi, 1985; Goetz and Cetta, 1985; Canario and Scott, 1987a, b; Scott and Canario, 1987; Thomas, 1994; Scott *et al.*, 1998). 17, 20ßP is known to be the most potent MIH in great majority of teleosts, particularly the salmonids. However, a number of other ovarian steroids including corticosteroids and deoxycorticosteroids have been found to have equal and in some cases even greater potency as MIS *in vitro* in several species (Goetz, 1983; Modesto and Canario, 1993; Trant and Thomas, 1988; Schoonen *et al.*, 1988; Kime *et al.*, 1991; Asahina *et al.*, 1992; Kime, 1993; Scott and Canario, 1987; Nagahama *et al.*, 1994; King *et al.*, 1994a, b). This observed diversity of MIH activity on species basis provided sufficient grounds to investigate which of the ovarian steroids may be the final MIH in the cyprinids selected for the present study. There is also evidence that the fully grown oocytes of some teleosts do not have the requisite competence to respond to MIH unless they have been primed by GTH (Lambert *et al.*, 1982; Kobayashi *et al.* 1988b; Zhu *et al.*, 1989; Patino and Thomas, 1990 a, b; Kagawa *et al.*, 1994). Exposure of the follicles to human chorionic gonadotrophin (hCG) *in vivo* or *in vitro* enhances their competence (Epler, 1981a, b; Epler *et al.*, 1987; Zhoa and Wright, 1985; Kime and Bieniarz, 1987; Patino and Thomas, 1990a, b, c; Thomas and Patino, 1991). The effect of hCG in promoting MIH activity by itself as well as in combination with different steroids was also tested *in vitro* in the present work on *C. watsoni* and *B. vagra*.

Elucidation of steroid biosynthesis has also been greatly facilitated by *in vitro* studies. Incubation of ovarian follicles in the presence of radiolabeled steroid substrates has yielded information that has enhanced our current understanding of the attendant enzyme pathways (fishes: Kime, 1993; Mammals: Gore-Langton and Armstrong, 1994). Progesterone (P_4), originally derived from cholesterol, is the first biologically important steroid, which is converted to 17α-hydroxyprogesterone (17-OHP) by 17α-hydroxylase. 17-OHP in turn yields androstenedione (AD) by cleavage of C-17, 20 carbon bond by the activity of 17, 20 desmolase (also called

lyase). Both 17α-hydroxylase and desmolase are part of single enzyme, cytochrome P450-c_{17}. 17-OHP is also converted to 17, 20βP in the ovarian granulosa by the enzyme 20β-hydroxysteroid dehydrogenase (20β-HSD). Testosterone (T) is derived from dehydroepiandrosterone (DHEA) or AD by the activity of 17β-hydroxysteroid dehydrogenase (17β-HSD). AD and T are then irreversibly converted, respectively, to estrone (E_1) and estradiol 17β (E_2) by aromatase P450, while AD and E_1 are reversibly metabolized to T and E_2, respectively, by isozymes of 17β-HSD (Gore-Langton and Armstrong, 1994). Besides the above enzyme systems other enzymes have also been identified in teleost ovaries such as reductases and hydroxylases that are responsible for inactivation of biologically active estrogens, androgens, progestogens and corticosteroids to their less active forms. These steroids are also inactivated through conjugation by sulphuronyl and glucuronyl transferases (Scott and Vermierssen, 1994). Besides facilitating excretion of steroids in urine and/or bile, they also seem to have a pheromonal role (Colombo *et al.*, 1980; Van den Hurk and Lambert, 1983; Van den Hurk *et al.*, 1987; Canario and Scott, 1989a; Scott and Vermierssen, 1994).

Several HPLC methods, based on both isocratic and gradient elution, have been described for the analysis of steroids (Shackleton, 1986; Robards and Towers, 1990; McDonald and Bouvier, 1995). The gradient elution usually allows separation of samples with large number of analytes but is limited by the reproducibility of the retention time that is usually less precise compared to the isocratic elution. However, this limitation is negligible when steroid concentrations are above the UV detection limit. Solid phase extraction provides clean extracts (Venkatesh *et al.*, 1989, 1992a; McDonald and Bouvier, 1995), circumventing the need of frequent regeneration of the column. Use of a 20 minute washing cycle at the end of each analytical run is sufficient to keep both the injector and the column sufficiently clean for numerous injections. The solid phase extraction is also very useful for simultaneous determination of both conjugated and unconjugated steroids as demonstrated by the accuracy of determination of the DHEA

(dehydroepiandrosterone) and its sulphated conjugates (Kime *et al.*, 1991). Sep-Pak 18 and reverse phase HPLC using gradient elution were, therefore, applied in the present study for separation and identification of steroids *in vivo* and *in vitro*.

Much of the focus on gonadal steroids in teleosts was initially directed at examination of steroid profiles of commercially valuable species, particularly the Salmoniformes (Reviews: Idler, 1972; Fostier *et al.*, 1983; Kime, 1993). The diversity of the teleostean group and the reproductive adaptations unique to its various taxa, however, necessitated a wider scope of research than was possible previously. Such a redirection of research in recent years has facilitated a far better understanding of the teleostean reproductive physiology and the endocrine events involved in cyclic development of the gonads round the year. It was with this background in view that the present work on *C. watsoni* and *B. vagra* was envisaged in an attempt to contribute to the wealth of information that became available in the eighties and nineties. An equally important consideration, in the context of local neglect of focus on a fundamentally important field of research, was the need to identify local species as models for future investigations that would be of value in efforts to control fish reproduction as part of aquacultural programs of research on commercially important species as well. Thus, in this investigation on C. *watsoni* and *B. vagra* the areas of focus were (1) study of seasonal profile of ovarian steroids in relation to development of the oocytes and annual reproductive cycles of these species, (2) determination of maturation-inducing potential of selected steroids and hCG *in vitro*, and (3) examination of the potential of the ovary of the *Cyprinion watsoni* to metabolize exogenous steroid substrates *in vitro*.

MATERIALS AND METHODS

Animals and Tissue Preparation

Barilius vagra and *Cyprinion watsoni*, belonging to the family Cyprinidae are commonly found in the hill streams of Islamabad where surface water temperature ranges during the year between 14°C in the coldest months and 29°C in the warmest months. Monthly samples of fish were collected with cast nets from Ramli stream in Islamabad. They were transported live to the experimental fish laboratory of the Department of Biological Sciences and sacrificed on the same day for seasonal profile and stocked for other studies for one week for acclimation to the ambient laboratory conditions in glass aquaria containing aerated water. They were given tropical fish food twice daily.

On the day of sacrifice, the total length and body weight of individual fish were recorded. The specimens of *C. watsoni* ranged between 10-14 cm in total length and weighed between 11-29 g., those of *B. vagra* ranged between 7-10 cm in total length and weighed between 3-5 g. Following sacrifice of the fish by decapitation, entire ovaries were dissected out, cleaned free of fat and weighed to the nearest mg on a Mettler balance. The ovarian weight and body weight were used for calculating gonadosomatic index (GSI = Ovarian weight as percent of body weight). For histological assessment of oocyte stages, one of the two ovaries from individual specimens was fixed immediately in aqueous Bouin's fixative for 24-48 hr. The other ovary from individual fish were pooled on monthly basis, weighed to obtain the requisite quantity (2-5 g) for analysis of steroids *in vivo* by HPLC and saved in a refrigerator at -20°C.

Light microscopy

The ovaries, fixed in aqueous Bouin's, were rinsed briefly in tap water and dehydrated in ascending ethanol series, cleared in benzene and embedded in paraffin wax. Tissue blocks were sectioned with a Cambridge Rotary Microtome at a thickness of 6-8 um. The sections were affixed to precleaned albuminized glass slides and

stained with haematoxylin and eosin. Microscopic examination was carried out under a research microscope (Optiphot Research Microscope, Olympus). Histological details and morphometric data in combination with macroscopic features of the gonads were used to determine the stage of development of the ovaries. Measurement of the oocyte diameter was made by a precalibrated ocular micrometer in order to obtain their mean size.

Chemicals and *In Vitro* Study

The steroids used in this study were estriol (E: estra-1,3,5 (10)-triene-3-16α, 17β-triol), estrone (E_1: estra-1,3,5 (10)-triene-3-ol-17-one), estradiol 17β (E_2 : estra-1,3,5 (10)-triene-3,17β-diol), testosterone (T:17β-hydroxy-4-androsten-3-one), androstenedione (AD: 4-androstene-3, 17-dione), 11β-hydroxyandrostenedione (11B-OHA: 11β-hydroxy-4-androstene-3, 17-dione), 19-hydroxyandrostenedione (19-OHA: 19β-hydroxy-4-androstene-3, 17-dione), progesterone (P_4: pregn-4-ene-3, 20-dione), 17-α-hydroxyprogesterone (17-OHP: 17α-hydroxypregn-4-ene-3, 20-dione), 17α-20β-dihydroxypregn-4-ene-3-dione (17,20βP: 17, 20β-dihydroxypregn-4-ene-3-one), 11-deoxycorticosterone (DOC: 21-hydroxypregn-4-ene-3,20-dione), corticosterone (B: 11β, 21-dihydroxypregn-4-ene-3, 20-dione), cortisol (F: 11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione) and aldosterone (ALDO: 11β, 21-dihydroxypregn-4-ene-3, 20-dion-18-al). These were received as a gift from Dr. Afzal Mehdi, Research Associate, Nortre Dame Hospital, University of Montreal, Quebec, Canada. 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20ßP) and human chorionic gonadotropin (hCG) were purchased from Sigma Chemical Co (St. Louis Missouri, USA). Two milligram of each steroid was dissolved in 2ml of methanol to make stock solutions from which three dilutions of 1.0, 0.1 and 0.01ug/ml of incubation media were achieved.

For *in vitro* study, mature fish were sacrificed in late March to early April as described above and the ovaries thus obtained were placed in ice-cold Basic Salt Solution (BSS) and reduced to small pieces. Individual ovarian follicles were separated using fine forceps without disrupting the integrity of the thecal and

granulosa layers. A sub sample of the 20 largest ovarian follicles was treated with oocyte clearing fixative (Zhoa and Wright, 1985), which revealed that such follicles contained oocytes with mostly central to nearly central germinal vesicle (CGV).

The culture techniques for *in vitro* incubation of the ovarian follicles were adapted from Upadhyaya and Haider (1986) with some modifications. The culture medium (BSS) consisted of 3.7 g NaCl, 0.32 g KCl, 0.16 g $CaCl_2$, 0.1 g NaH_2PO_4, 0.16 g $MgSO_4.7H_2O$ and 0.8 g glucose in one-liter of double distilled water. The medium was autoclaved, cooled and its $_pH$ was adjusted to 7.5 with sterilized 1N sodium bicarbonate ($NaHCO_3$). Bacterial and fungal contamination was controlled with Penicillin-Streptomycin (100 IU/ml) and Fungizone (2.5 ug/ml). The medium was sterile filtered through a Millipore filter of GS 0.22 um (Bedford, Mass, USA). Filtration was usually done in a Laminar Flow Cabinet. If necessary the medium was stored in a refrigerator at 4°C.

In vitro experiments were conducted inside a sterile room in the Tissue Culture Laboratory of the Department of Biology. All glassware and dissecting instruments were immersed in weak chromic acid overnight, rinsed with tap water, washed with 7x detergent and once with 70% ethanol, oven dried and autoclaved. Before starting the experiments, all glassware was exposed to ultraviolet light for one to two hours. Samples of 25-35 ovarian follicles were transferred under the Laminar Flow Cabinet to individual culture tubes containing 3.0 ml fresh culture medium. Test steroids were added to each dish with a micro syringe to obtain concentrations of 1.0, 0.1 and 0.01ug/ml of the culture medium. The control cultures received an equal volume of 96% ethanol or saline depending on the solvent used for hormone dilution. Viability of the oocytes was checked in preliminary tests by Trypan Blue as well as by estimation of total protein (Lowry *et al.*, 1952) in samples of cultured oocytes at various time intervals. According to this test, viable oocytes remain clear following the treatment while the dead oocytes take up the stain. The incubations for each test system were run in triplicate. The replicate cultures were maintained at 22 ± 0.2^0C in

a Sanyo temperature-controlled incubator (Sanyo, MIR 251) for 24, 48, 72 hr each. Upon completion of the incubations, the incubation medium was aspirated and stored at -20^{0}C, and the follicles were examined for germinal vesicle breakdown (GVBD) by treating the follicles with egg clearing solution (95% ethanol, 10% formalin and acetic acid in a ratio of 95:10:5) (Zhoa and Wright, 1985), and the number of mature oocytes was counted under an inverted microscope (Nikon, Diaphot TMD) to calculate the % age GVBD.

Extraction of steroids from the ovarian tissue and the incubation medium

The extraction procedure was based on the methods described by Huang *et al.* (1989), Venkatesh *et al.* (1989, 1992a), Kime *et al.* (1991), Scott and Canario (1992) and Khan, (1996), with some modifications. The ovarian tissue and the follicles (*in vivo* and *in vitro* study, respectively) were homogenized, sonicated and digested with 0.5N NaOH at 65^{0}C for 30 minutes. The steroids were extracted with 8 volumes of diethyl ether. Following evaporation of the organic phase under nitrogen, it was redissolved in 5ml of distilled water and the solution was applied to a Sep- Pak C 18 cartridge (Millipore Corporation, Milford, MA, USA) as described below.

The steroid hormone metabolites generated by the ovarian tissue and released in the incubation replicates were directly applied to a Sep-Pak C 18 column that had been primed by washing with 5 ml of methanol and 5 ml of distilled water. The column was then washed with 5 ml of water followed by 5 ml of hexane. Subsequently, the free steroid hormones were eluted from the column by using 5 ml of diethyl ether and the conjugated steroids were eluted with 5 ml of 100% methanol. The ether and methanol extracts were dried separately under nitrogen at 45^{0}C. The free fractions (unconjugated metabolites) were pooled and dissolved in 200ul of acetonitrile. A 20-ul aliquot of this mixture was injected on to the HPLC column. The dried, conjugated steroid fraction was processed by enzyme hydrolysis and acid solvolysis to obtain free steroids for HPLC (see below).

Acid solvolysis of sulphate

The dried methanol fraction, containing the conjugated steroids, was incubated overnight at 45°C in 5 ml of trifluoroacetic acid/ethyl acetate (TFA/EA (1/100; v/v) to convert the sulphated steroid to its free form. The solvent was then evaporated to dryness and redissolved in 5 ml of water. The free (formerly sulphated) and the glucuronidated steroids were concentrated and separated as described above, using a Sep-Pak C_{18} cartridge. The solvolyzed steroids were recovered with 5ml of diethyl ether. The glucuronide conjugates were eluted with 5 ml of 100% methanol as above and subjected to enzyme hydrolysis.

Enzyme hydrolysis of glucuronides

The dried methanol fraction containing conjugated steroids was reconstituted with 1.0 ml of 0.5 M sodium acetate buffer (PH 5.0) and hydrolyzed at 37^0C using 20 ul of β-glucuronidase (Snail extract containing 2000 units of enzyme activity). The hydrolyzed steroids were added to 5 ml of water and extracted using Sep-Pak C 18 column and 5 ml diethyl ether (see above).

Steroid metabolites in incubation medium (Recovery study)

In order to determine the efficiency of the extraction procedure 14 unlabeled steroids (2.0 ug each) were mixed and dissolved in 5 ml of water before extraction. The mixture was separated on Sep-Pak C 18 cartridge, eluted with diethyl ether, dried, reconstituted with acetonitrile or methanol and resolved by HPLC. More than 95% of the steroids were recovered in fractions.

HPLC methodology

All chemical used were "reagent grade" or chromatography grade". Methanol and acetonitrile were purchased from BDH. Solvents for HPLC were degassed under vacuum immediately before use. Water was deionized and glass distilled. The stock steroid standards (2 mg/2 ml) were made up in methanol and

diluted to obtain working standards. The stock and working standards were stable for at least six months at -20°C.

The HPLC system (LC- 6A, Shimadzu Co., Kyoto, Japan) had a two-pump, variable wavelength detector with UV signals integrated on line with chromatopack C-R3A data system. Reverse phase chromatography with gradient elution was selected because of the wide range of polarities of the steroids that needed to be separated in the samples. This system has the advantages of high selectivity and rapid re-equilibration. The column used was 4.6 x 250 mm, stainless steel commercially packed with Zorbax-ODS. It was empirically found that a concave exponential gradient of methanol-water starting with 40% methanol and ending with 100% organic solvent gave the most satisfactory separation of the ovarian steroids that could be achieved at 45°C within 50 min at a rate of 1.1 ml/min. By using this mobile phase, testosterone was occasionally mixed up with 17,20αP. The problem was overcome by using an acetonitrile-water multistep gradient system consisting of 20, 28, 45, 70, 100% acetonitrile in water at elution times 0, 15, 40, 50, 55 min, respectively. Retention times for each steroid were determined by injecting 50 ng of each standard (20-ul aliquots), with absorbance monitored at 254 nm and 280 nm. Quantification of each steroid was based on peak area calculated as peak height (cm) x peak width at half peak height (cm). Drift in retention times of the steroids was ascertained before each day's analysis by one of two ways: injection of the mixtures of steroid standards or ovarian samples, spiked with progesterone as reference steroid for calculating the relative retention times of the other steroids.

The methodology developed enabled extraction and separation of the following reference steroids: estriol (E), estrone (E_1), estradiol (E_2), testosterone (T), androstenedione (AD), 11ß-hydroxyandrostenedione (11ß-OHA), 19-hydroxyandrostenedione (19-OHA), progesterone (P_4), 17α-hydroxyprogestrone (17-OHP), 17α, 20ß-dihydroxy-4-pregnene-3-one (17, 20ßP), 11-deoxycorticosterone

(DOC), corticosterone (B), cortisol (F) and aldosterone (ALDO). The retention times for these steroids are shown in Figs. 1, 2, 3.

Statistical analysis

The data of the *in vivo* steroid profile and the *in vitro* recovery study were analyzed by calculating standard errors of the means. The results of *in vitro* maturation study were subjected two-way analysis of variance (Anova) and Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1996).

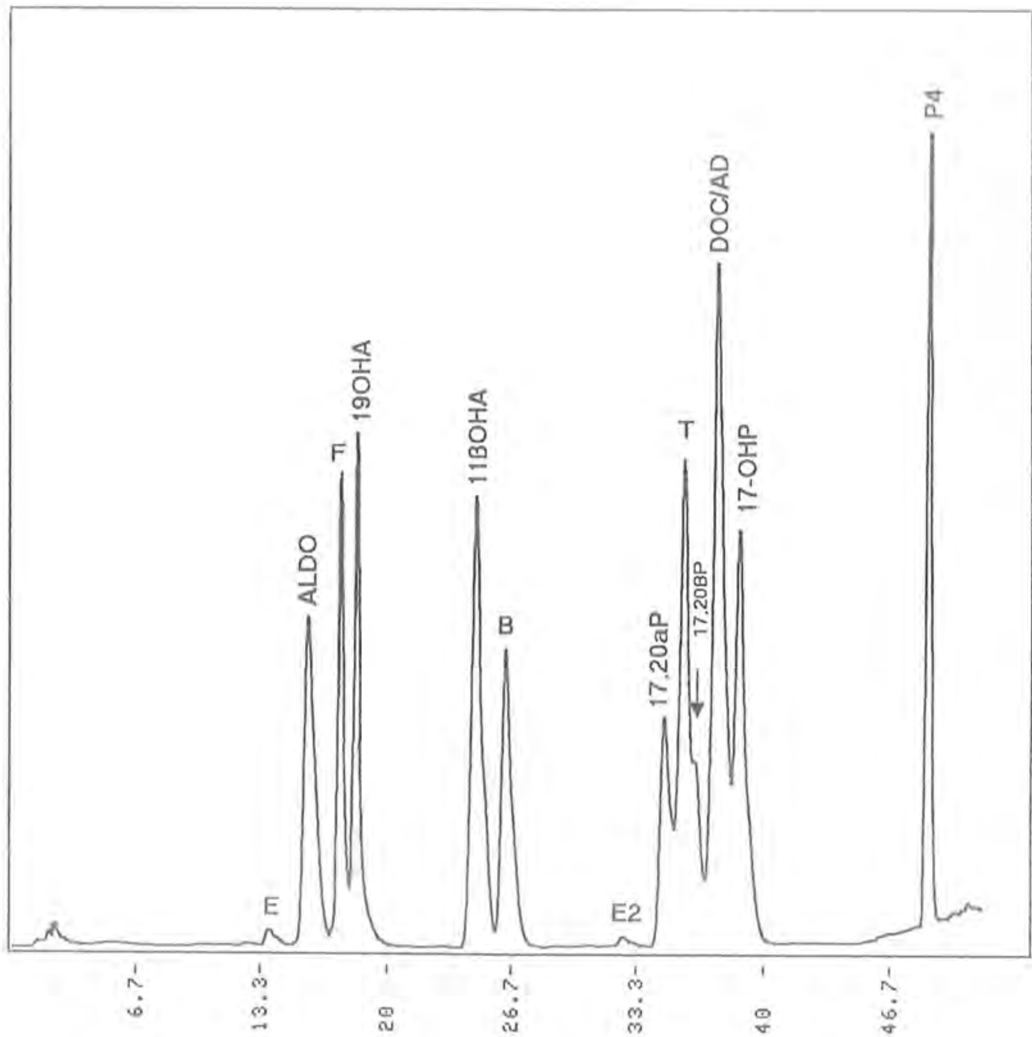

Fig 1. Chromatographic separation of steroid standards (200ng/20ul each) on Zorbax-ODS using acetonitrile-water gradient. UV detection at 254nm.

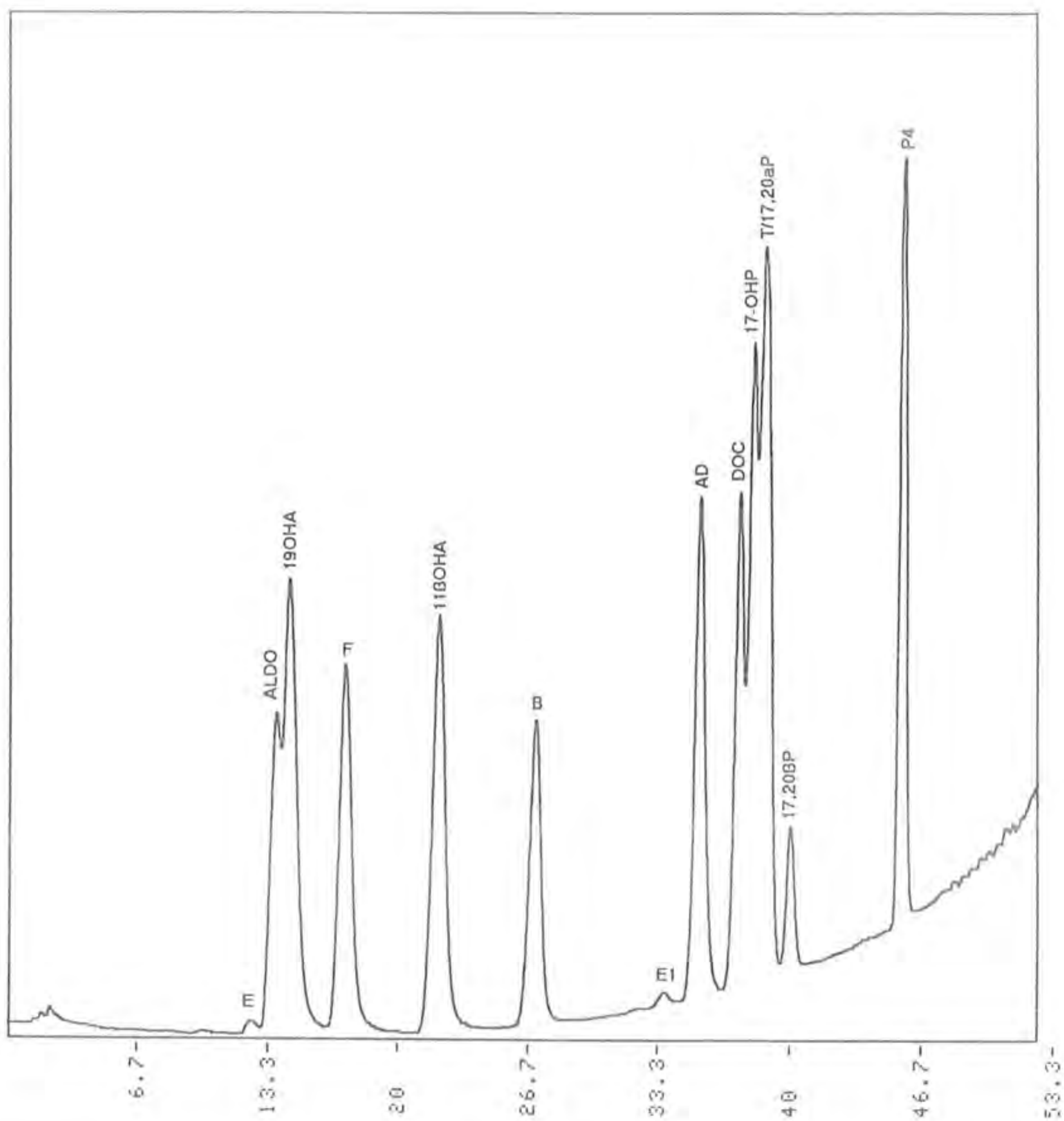

Fig 2. Chromatographic separation of steroid standards (200ng/20ul each) on Zorbax-ODS using methanol-water gradient. UV detection at 254 nm.

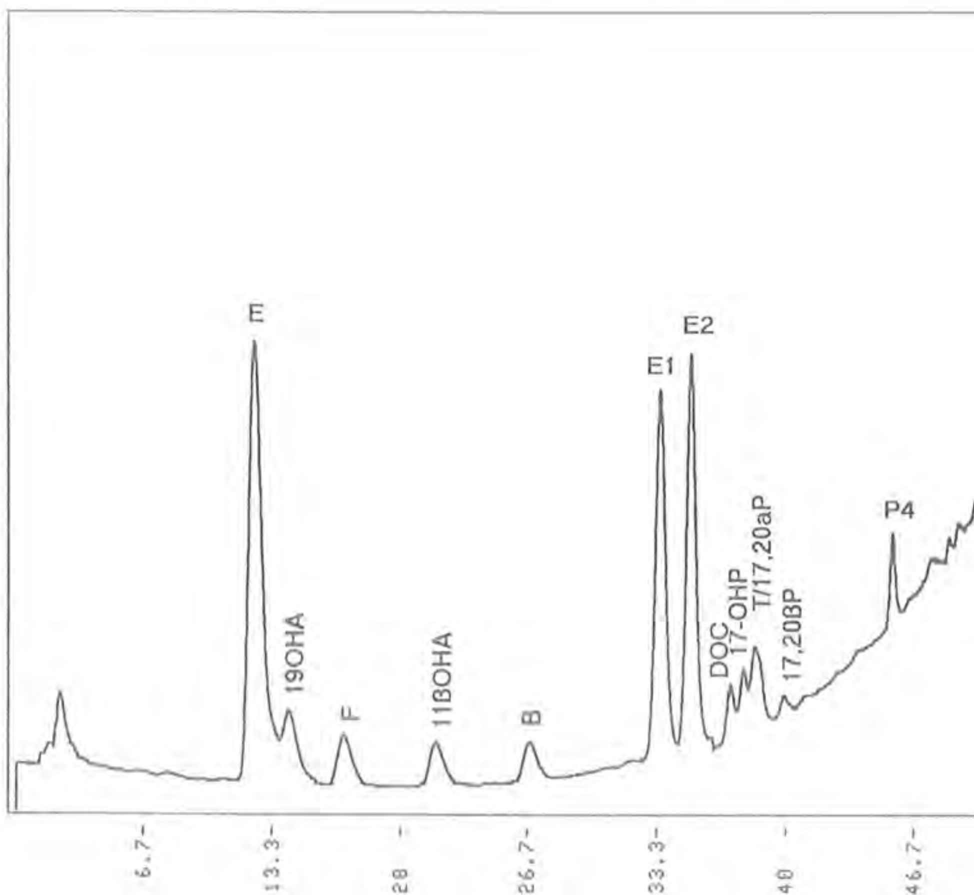

Fig 3. Chromatographic separation of steroid standards (200ng/20ul each) on Zorbax-ODS using methanol-water gradient.UV detection at 280 nm.

RESULTS

Seasonal changes in ovarian cycle

Table 1 describes classification of stages of the seasonal ovarian cycle of *Cyprinion watsoni* and *Barilius vagra*. Both species showed asynchronous ovarian development with oocytes in various stages of development at any one time. The classification was based on groups of the largest oocytes. Stage I represented ovaries in December and January in previtellogenic phase of recovery from the previous spawning. Most of the oocytes in the ovaries in February, March and April were in primary, secondary and tertiary vitellogenic stages of growth and constituted, respectively, Stages II, III and IV. In May, the ovaries were ripe and contained hyaline eggs as well as oocytes in various earlier stages. The spawning period was long extending from May to August. Spent ovaries representing the postspawning Stage VI in September to November were characterized by follicular atresia, internal hemorrhage and residual follicles.

Seasonal changes in Gonadosomatic Index (GSI)

The mean GSI of *C. watsoni* and *B. vagra* (Figs.4,5) was low in December to February, increased (vitellogenic growth) to a peak in April and then declined gradually during the spawning months to the lowest postspawning level in November. In *B. vagra*, the GSI was similar but showed a significant fall in June and a second peak in July beyond which it continued to decline to the low values of September to November (Fig. 5).

Seasonal profile of Steroid hormones

The steroids were determined monthly throughout the annual reproductive cycle. Several estrogens, progestogens, androgens and corticosteroids were identified in the ovary of adult *Cyprinion watsoni* and *Barilius vagra*.

Estrogens

The ovarian titers of estriol (E), estrone (E_1) and estradiol 17β (E2) in the two species are summarized in Tables 2 and 3 (Figs. 6, 7).

Table 1. Maturity stages of ovaries in *Cyprinion watsoni* and *Barilius vagra*.

Stage	Appearance	Oocyte Stage (largest oocytes)	Histological condition
I Recovering Dec/Jan	Thread-like	Perinucleolus stage (<70 um)	Virgin and recovering ovaries, oogonial nest, non yolky oocytes
II Onset of vitello-genesis, Jan/Feb	Granular ovaries, light yellow	Primary yolky stage (mean dia 180 um)	Preparatory phase, cortical alveolar and early vitellogenic stages predominate
III Advanced vitello-genesis, March	Ovaries enlarged, bright yellow	Secondary yolky and some tertiary yolky stage oocytes (mean dia 390 um)	Yolky oocytes, secondary yolky stages predominate with some perinucleolar and tertiary yolky stage follicles
IV Completion of vitellogenesis, April	Further increase in size	Tertiary yolky stage (mean dia 480 um)	Yolky deposition complete, tertiary yolky stage predominates with central germinal vesicle, some migratory stage and secondary stage follicles also present
V Mature, May-August	Hyaline eggs, flow freely with pressure	Ripe ovaries (>500 um)	Migratory stage follicles and some lacking germinal vesicle predominate, other stages also exist
VI Spent Sep-Nov	Dirty brown with residual eggs	Follicular atresia	Collapsed, postovulatory follicles, internal hemorrhage, atretic follicles also present.

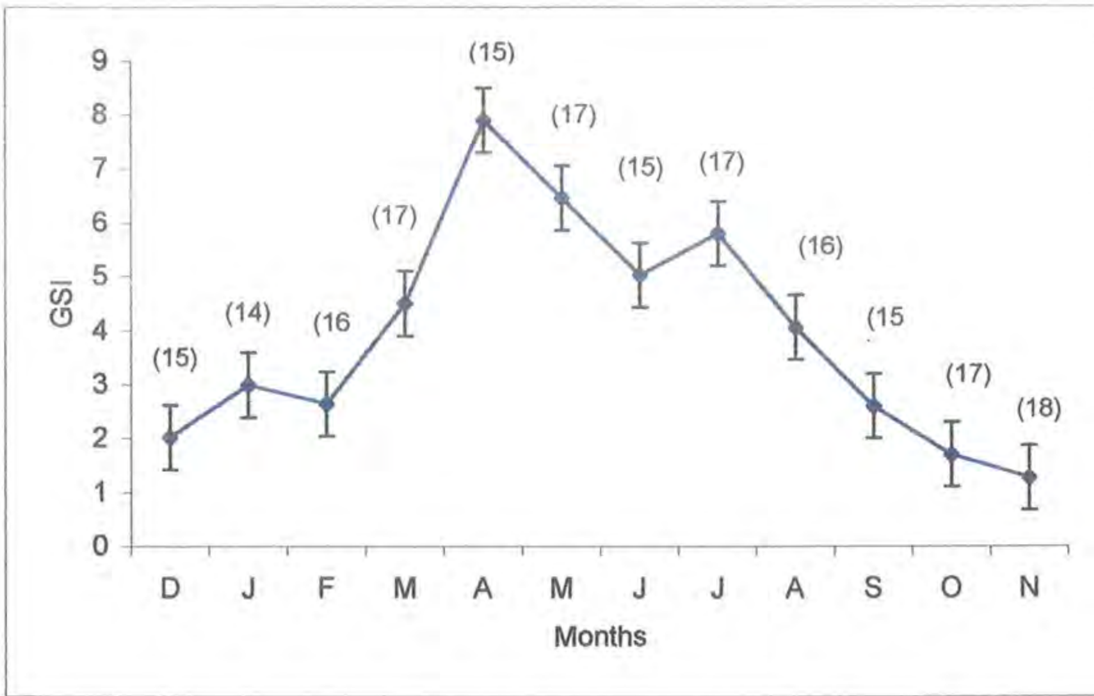

Fig 4. Seasonal variation in the mean gonadosomatic index (GSI) ± S.E in female *Cyprinion watsoni.* Figures in parenthesis are number of animals.

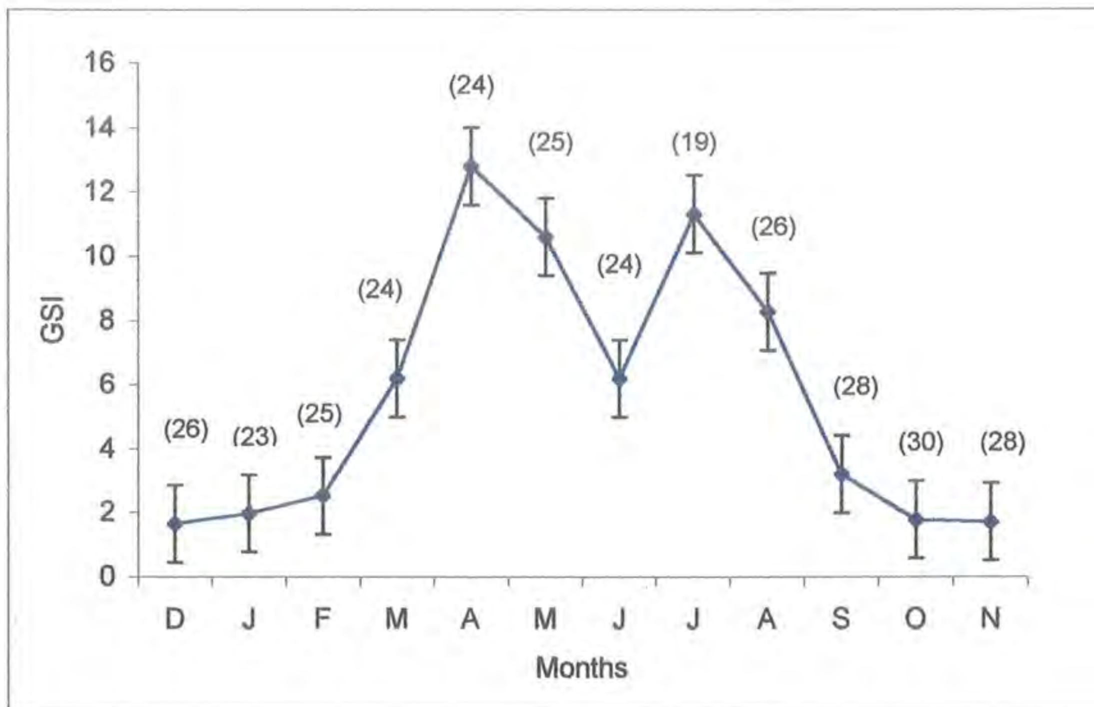

Fig 5. Seasonal variation in the mean gonadosomatic index (GSI) ± S.E in female *Barilius vagra.* Figures in parenthesis are number of animals.

Table 2. Seasonal profile of ovarian estrogens (ng/100mg ± S.E) in *Cyprinion watsoni.*

	E	E1	E2
December	0.80±0.3	1.26±0.41	1.51±0.30
January	1.47±0.37	3.27±0.72	2.66±0.66
February	3.13±0.26	3.98±1.12	4.59±0.35
March	6.19±0.81	6.52±1.49	10.05±1.51
April	0.85±0.43	4.19±0.61	4.30±1.09
May	0.81±0.45	3.74±0.88	1.33±0.69
June	1.94±0.09	1.78±0.46	2.16±0.21
July	1.46±0.14	1.09±0.17	1.34±0.14
August	0.59±0.29	0.68±0.25	1.32±0.47
September	n.d	n.d	n.d
October	n.d	n.d	n.d
November	n.d	n.d	n.d

Table 3. Seasonal profile of ovarian estrogens (ng/100mg ± S.E) in *Barilius vagra.*

	E	E1	E2
December	0.72±0.3	1.54±0.38	0.97±0.33
January	1.76±0.64	3.67±1.20	2.79±0.69
February	3.35±1.30	3.17±0.19	7.29±2.60
March	3.20±0.70	9.17±3.69	6.7±0.596
April	3.14±0.64	4.94±0.99	2.90±0.42
May	1.67±0.85	3.47±0.64	1.96±0.19
June	1.34±0.26	2.37±0.15	2.46±0.26
July	1.10±0.17	2.10±0.21	1.86±0.09
August	n.d	n.d	n.d
September	0.11±0.02	0.48±0.14	0.47±0.12
October	n.d	n.d	n.d
November	n.d	n.d	n.d

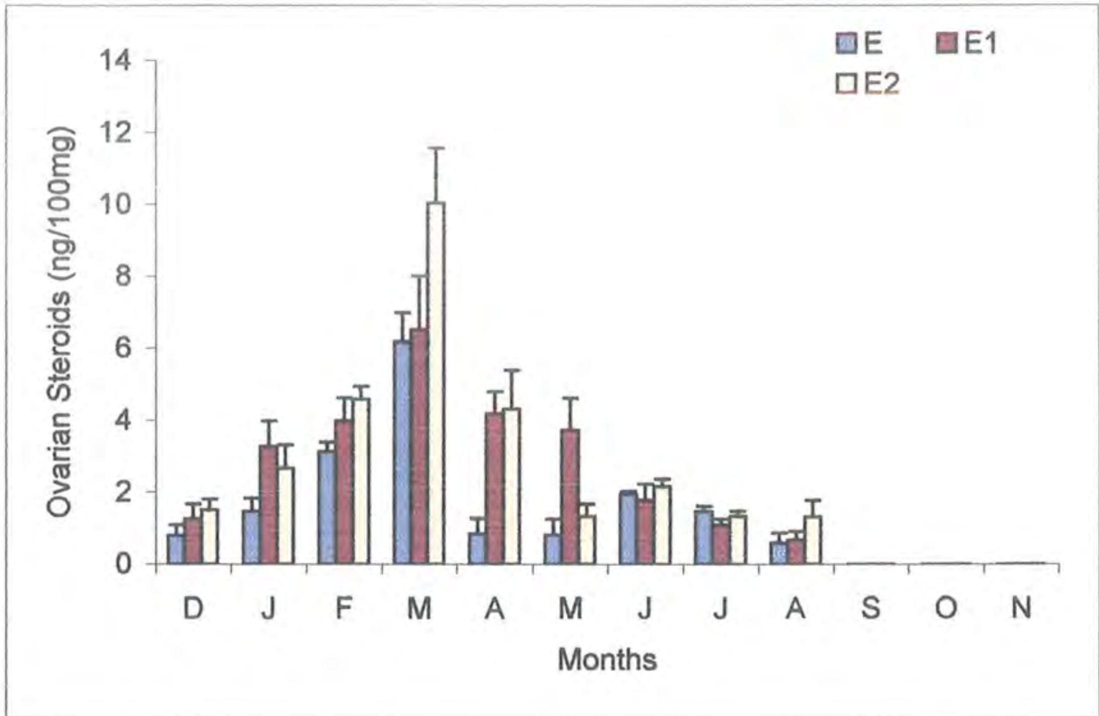

Fig 6. Seasonal variation in ovarian estrogens (*Cyprinion watsoni*).

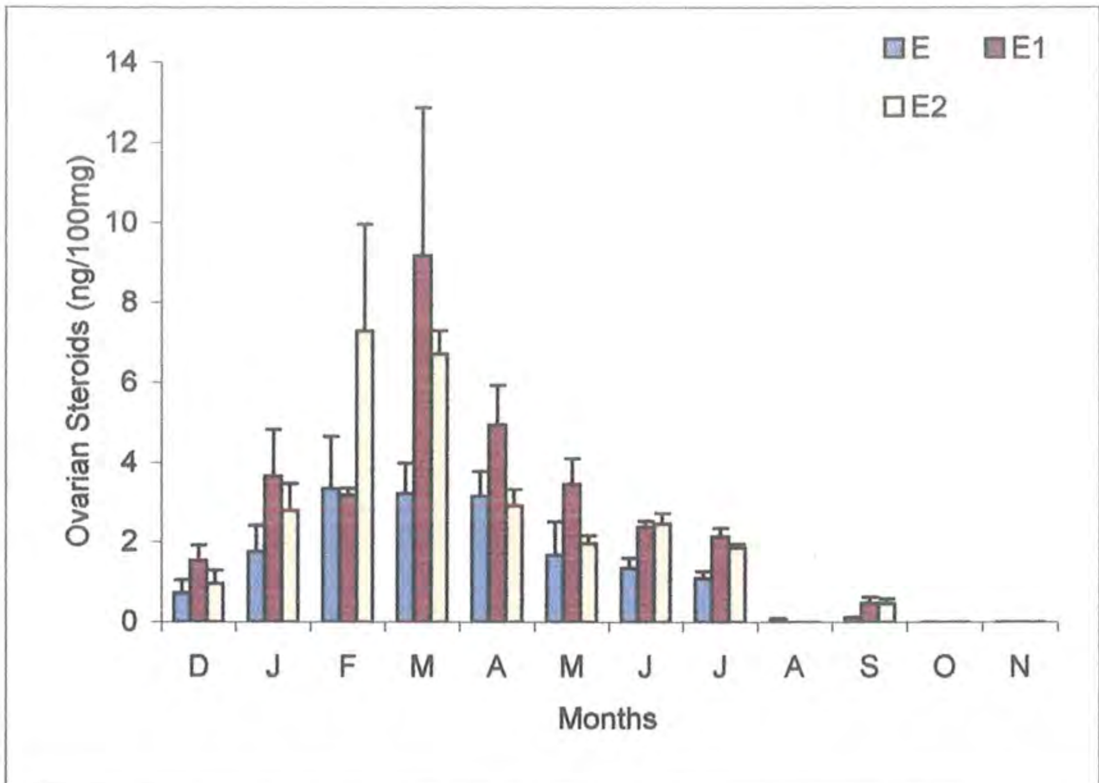

Fig 7. Seasonal variation in ovarian estrogens (*Barilius vagra*).

Table 4. Seasonal profile of ovarian androgens (ng/100mg ± S.E) in *Cyprinion watsoni.*

	T	AD	19OHA	11ßOHA
December	0.98±0.19	0.91±0.45	0.74±0.33	1.45±0.56
January	2.16±0.34	1.20±0.23	1.97±0.17	0.50±0.02
February	2.63±0.28	4.76±0.35	1.85±0.62	1.86±0.15
March	6.79±1.35	5.30±1.36	2.46±0.79	3.83±1.27
April	4.29±1.06	3.16±0.68	1.89±0.24	2.22±0.46
May	2.03±0.61	1.68±0.45	0.81±0.14	1.06±0.18
June	1.53±0.35	1.66±0.27	0.84±0.27	4.63±0.50
July	1.04±0.35	0.50±0.04	1.38±0.34	1.88±0.50
August	1.96±0.50	1.16±0.40	4.24±1.46	3.92±1.20
September	0.52±0.46	0.14±0.04	n.d	0.14±0.12
October	0.90±0.16	0.34±0.04	0.18±0.18	0.53±0.29
November	0.39±0.11	0.52±0.01	0.07±0.07	0.998±0.4

Table 5. Seasonal profile of ovarian androgens (ng/100mg ± S.E) in *Barilius vagra.*

	T	AD	19OHA	11ßOHA
December	0.61±0.09	1.22±0.43	0.62±0.08	0.99±0.11
January	1.86±0.26	2.18±0.17	1.49±0.32	1.45±0.19
February	3.85±0.24	3.00±0.73	0.47±0.17	2.70±0.90
March	3.14±0.17	3.58±0.62	5.69±1.00	8.41±1.52
April	1.99±0.24	2.18±0.17	2.94±0.55	2.71±0.74
May	2.76±1.04	2.81±0.67	3.05±1.02	3.09±1.19
June	0.89±0.08	1.13±0.12	0.97±0.12	2.08±0.83
July	1.31±0.23	1.17±0.04	0.76±0.06	0.92±0.02
August	0.70±0.06	0.84±0.17	0.69±0.01	1.12±0.05
September	0.64±0.16	0.75±0.46	0.85±0.82	0.50±0.05
October	0.33±0.06	0.25±0.06	0.44±0.08	0.72±0.19
November	0.29±0.05	0.22±0.03	0.34±0.07	0.69±0.07

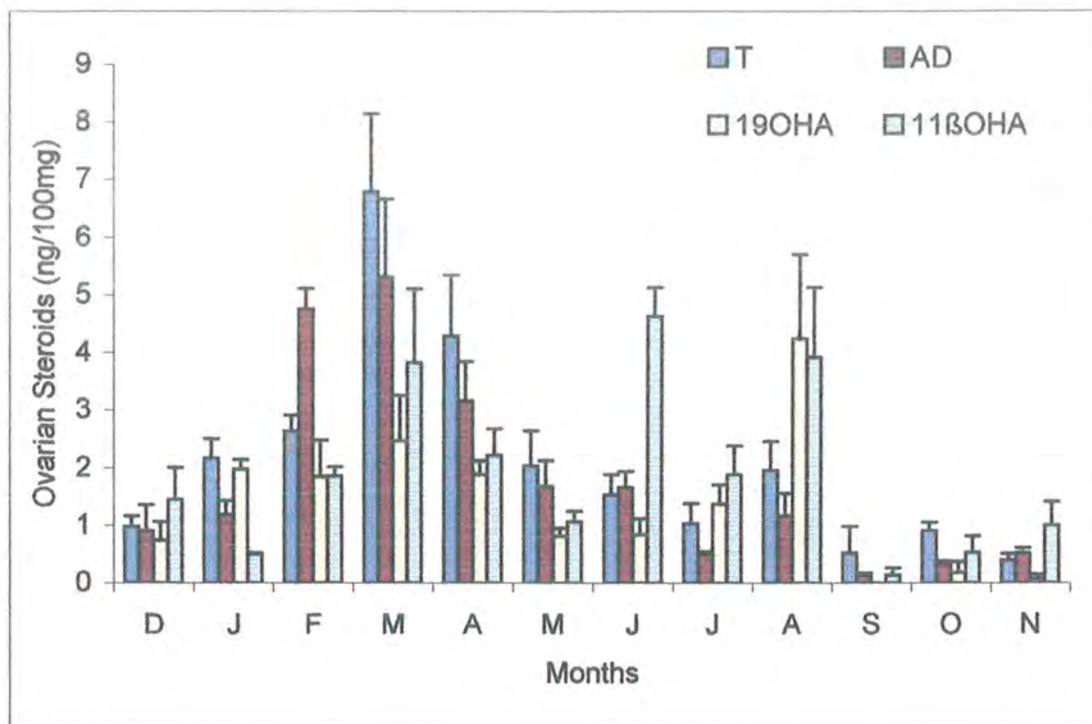

Fig 8. Seasonal changes in ovarian androgens (*Cyprinion watsoni*).

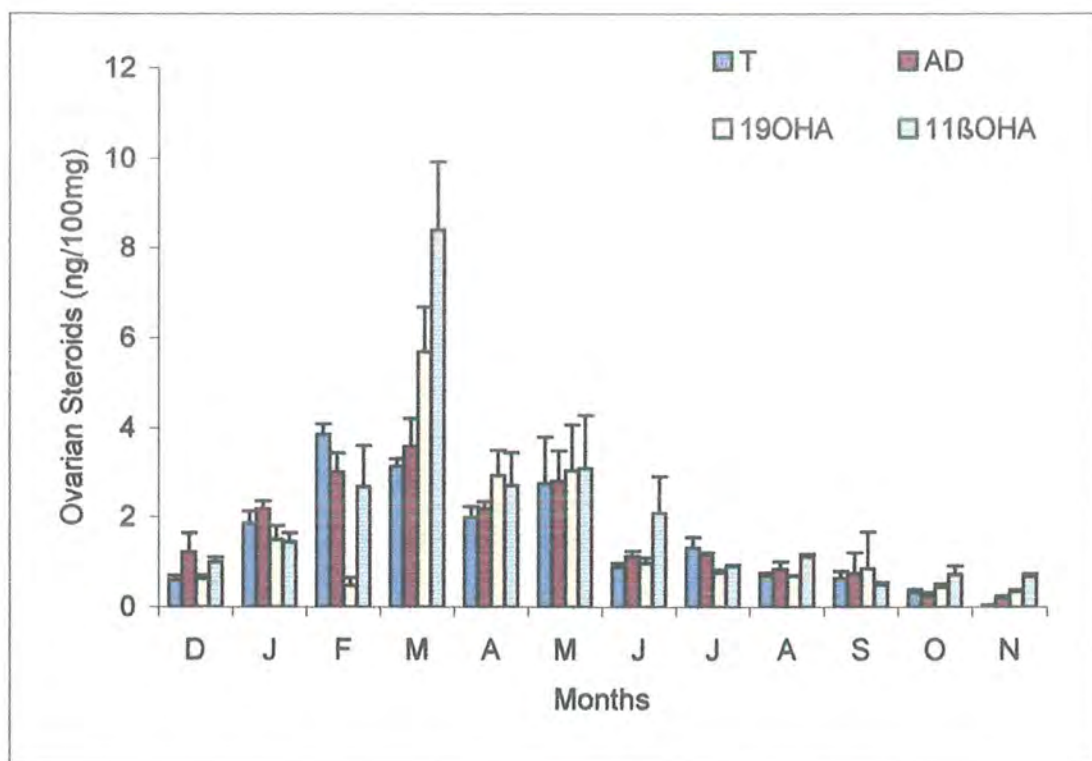

Fig 9. Seasonal changes in ovarian androgens (*Barilius vagra).*

E, E_1 and E_2 were detected in *C. watsoni* in December to August and coinciding with ovarian recrudescence and vitellogenesis (Table 2, Fig. 6). The levels of the three estrogens (ng/100 mg) varied, respectively, from 0.59 (August) to 6.2 (March), 0.68 (August) to 6.52 (March) and 1.32 (August) to 10.5 (March). However, E_1 persisted at relatively high levels for a longer duration (February to May). These estrogens were undetectable in September to November. It is noteworthy that while E dropped sharply and transiently in April, E_1 declined in May to only 50% of its value in March. A significant level of E_2 was evident even in June.

The levels of E, E_1 and E_2 in *B. vagra*, ranged, respectively, from 0.11 (September) to ~3.00 (February to April), 0.48 (September) to ~9.0 (March) and 0.47 (September) to ~7.0 (February/March) ng/100 mg (Table 3, Fig.7). In August and September, their levels were close to or below the detectable limit. The decline in E in June was about 50% of its value in March. E_1 and E_2 again persisted at noticeably high levels even in July.

In both species, the levels of these steroids were lowest in the postspawning season (spent ovaries), increased maximally corresponding with vitellogenic progress and declined gradually in the spawning months.

Androgens

Four androgens, testosterone (T), androstenedione (AD), 19-hydroxyandrostenedione (19-OHA) and 11β-hydroxyandrostenedione (11β-OHA) were identified in the ovaries of the two species throughout the year (Tables 4, 5; Figs. 8, 9). A few additional peaks of androgens were also detected but could not be identified due to lack of the desired standards. The lowest levels of the androgens in both species prevailed during the postspawning months of September, October and November.

In *C. watsoni*, the level of T started increasing in January, reached the maximum value in March (6.19 ng/100 mg) followed by gradual decline in the spawning season

Table 6. Seasonal profile of ovarian progestogens (ng/100mg ± S.E) in *Cyprinion watsoni*.

	P4	17-OHP	17,20ßP
December	1.59±0.45	0.65±0.40	0
January	2.61±0.32	1.42±0.67	0.54±0.03
February	4.45±0.70	3.78±1.34	0.78±0.08
March	4.33±0.90	5.93±1.68	3.86±0.25
April	3.12±0.43	7.05±1.09	15.72±4.24
May	2.13±0.37	3.84±1.92	11.89±5.32
June	1.14±0.22	4.24±1.57	6.82±0.30
July	0.69±0.13	1.19±0.59	0.93±0.08
August	2.05±0.74	1.55±1.03	1.26±0.74
September	0.59±0.26	0.20±0.30	n.d
October	0.22±0.13	0.11±0.06	0.11±0.11
November	0.66±0.35	0.63±0.37	n.d

Table 7. Seasonal profile of ovarian progestogens (ng/100mg ± S.E) in *Barilius vagra*.

	P4	17-OHP	17,20ßP
December	0.95±0.06	0.85±0.06	0.33±0.03
January	1.33±0.18	1.44±0.35	1.16±0.69
February	1.83±0.07	1.20±0.43	3.17±2.30
March	2.03±0.42	14.27±6.25	48.7±23.8
April	0.61±0.13	4.57±0.49	25.56±5.38
May	2.23±0.59	3.22±0.38	6.86±3.81
June	0.79±0.24	3.73±0.27	2.61±1.36
July	1.08±0.10	2.93±0.37	5.49±1.45
August	1.29±0.14	0.57±0.08	1.77±0.49
September	1.05±0.13	0.14±0.05	n.d
October	0.92±0.07	0.15±0.03	0.35±0.14
November	n.d	n.d	n.d

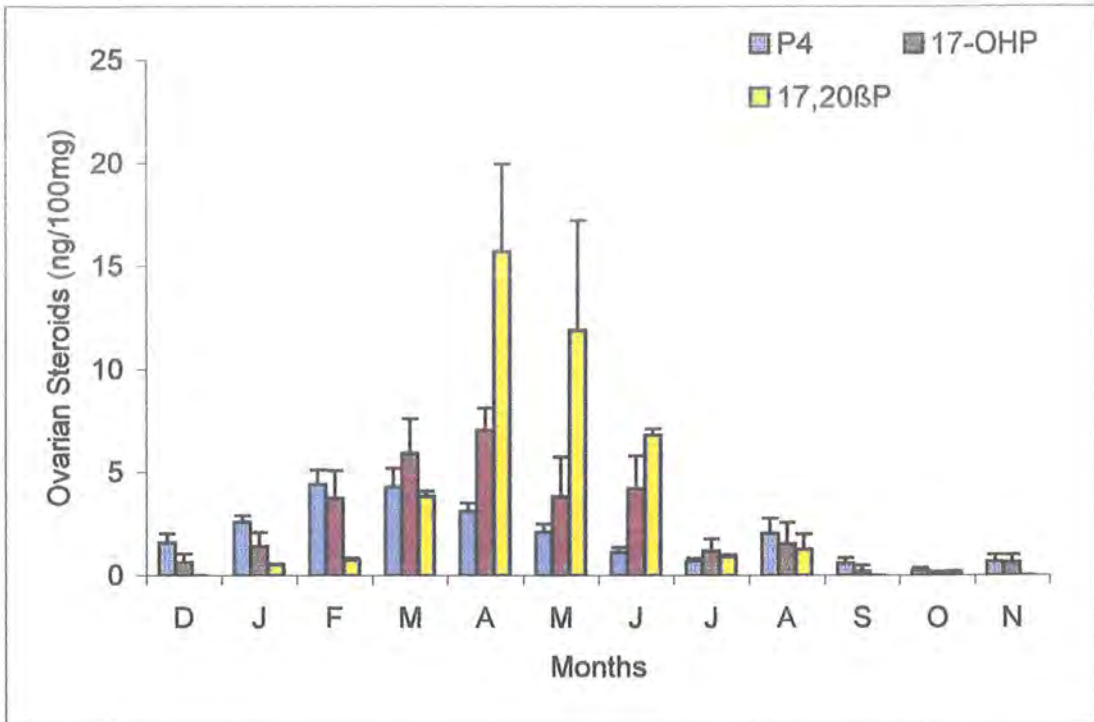

Fig 10. Seasonal variation in ovarian progestogens (*Cyprinion watsoni)*

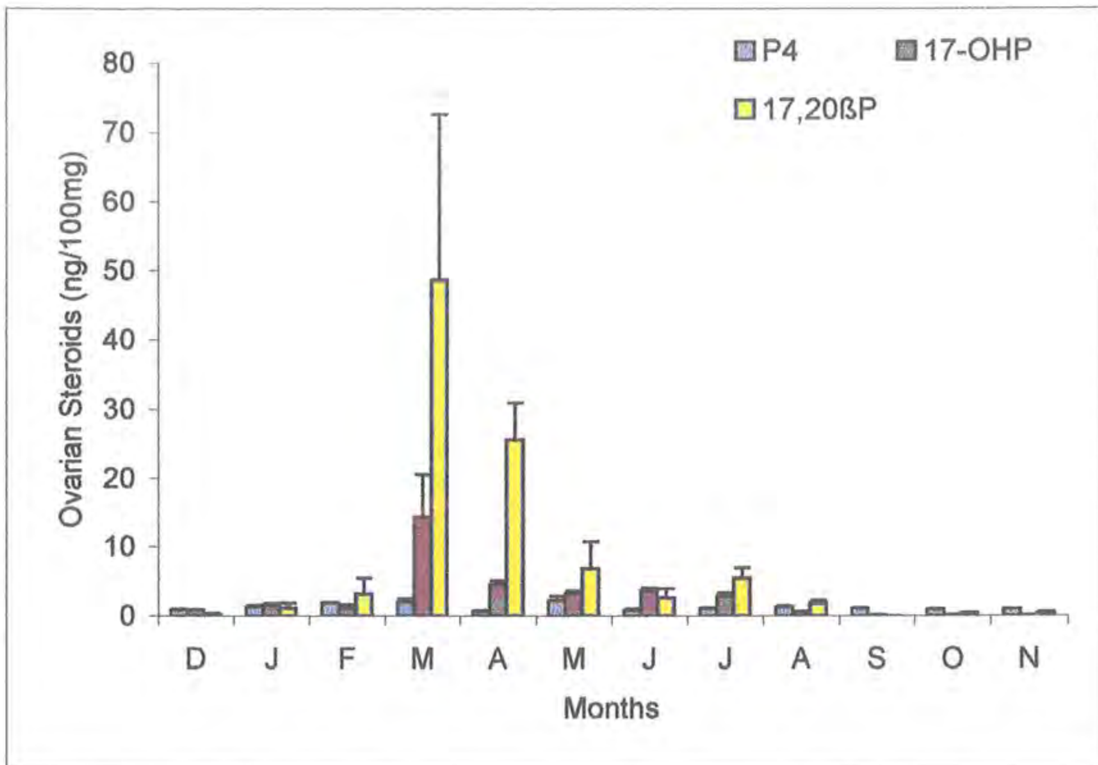

Fig 11. Seasonal variation in ovarian progestogens (*Barilius vagra)*

(Table 4, Fig. 8). AD showed a significant increase in February/March. Its level was 2-fold higher than that of T in February, becoming comparable to the level of T in March and noticeably higher in June compared to the postspawned fish. The level of 19-OHA increased to a peak in March, declined sharply in May and peaked again to its highest level in August. 11β-OHA reached peak levels intermittently in March, June and August.

In *B. vagra*, the concentration of only T, AD and 11β-OHA increased sharply over the December value in February (Table 5, Fig. 9). While T and AD remained at nearly the same level in March and declined in April, both 19-OHA and 11β-OHA increased to their highest values in March. Of the four androgens, the highest concentration was that of 11β-OHA. A sharp decline in the level of 19-OHA occurred in June (mid-spawning) and in 11β-OHA in July. The levels of the androgens remained low throughout the following postspawning months.

Progestogens

The chromatographic peaks of only three progestogens could be identified in both species (Tables 6, 7; Figs. 10, 11). In *C. watsoni*, progesterone (P_4) and 17-OHP were detectable throughout the year, whereas 17-20βP remained extremely low or below the detectable limit in the postspawning months of September, October, November and December (Table 6, Fig. 10). The levels of P_4 and 17-OHP were also extremely low in this species in the postspawning period of September to November. Increasing levels of P_4 and 17-OHP occurred beginning with December and January, respectively, and remained at peak levels in February/March (P_4) and March/April (17-OHP) before declining in the spawning season. 17-OHP was still high in June followed by a sharp decrease beginning in September only. A dramatic increase in 17, 20βP was recorded in March. It increased 5-fold in April (onset of maturation) when it achieved its highest level (16 ng/100 mg). It declined slightly in the spawning months of May and by about 50% in June, reaching its lowest values in the postaspawning season.

The seasonal profile of the progestogens in *B. vagra* was uniquely different from that of *C. watsoni* (Table 7, Fig. 11). The levels of P_4 were generally low throughout the year

Table 8. Seasonal profile of ovarian corticosteroids (ng/100mg ± S.E) in *Cyprinion watsoni*

	DOC	B	F
December	2.06±0.38	0.72±0.29	0.20±0.10
January	2.82±0.15	1.07±0.42	1.39±0.37
February	3.21±0.25	1.63±0.12	1.81±0.45
March	7.82±1.44	3.72±1.24	0.98±0.37
April	5.00±1.46	1.87±0.25	1.49±0.27
May	1.08±0.19	1.62±0.23	0.78±0.06
June	1.80±0.15	1.22±0.23	1.17±0.66
July	1.06±0.05	1.69±0.42	1.37±0.46
August	2.50±0.53	1.19±0.30	2.01±0.53
September	0.19±0.04	0.07±0.00	0.21±0.06
October	0.26±0.17	0.42±0.11	0.24±0.14
November	1.18±0.31	0.1±0.08	0.56±0.49

Table 9. Seasonal profile of ovarian corticosteroids (ng/100mg ± S.E) in *Barilius vagra*

	DOC	B	F
December	1.98±0.46	0.43±0.07	0.40±0.07
January	1.56±0.15	1.28±0.79	0.71±0.09
February	5.70±2.80	1.96±0.79	0.75±0.06
March	4.56±1.20	6.10±1.12	4.20±0.76
April	6.27±0.67	3.86±0.93	2.74±0.76
May	7.09±1.95	3.95±1.26	2.19±0.84
June	2.67±0.43	1.89±0.60	0.69±0.09
July	4.70±0.61	1.66±0.06	0.69±0.01
August	1.13±0.15	1.06±0.18	0.57±0.09
September	0.24±0.09	0.25±0.06	0.23±0.16
October	1.13±0.45	1.10±0.23	0.67±0.21
November	n.d	n.d	n.d

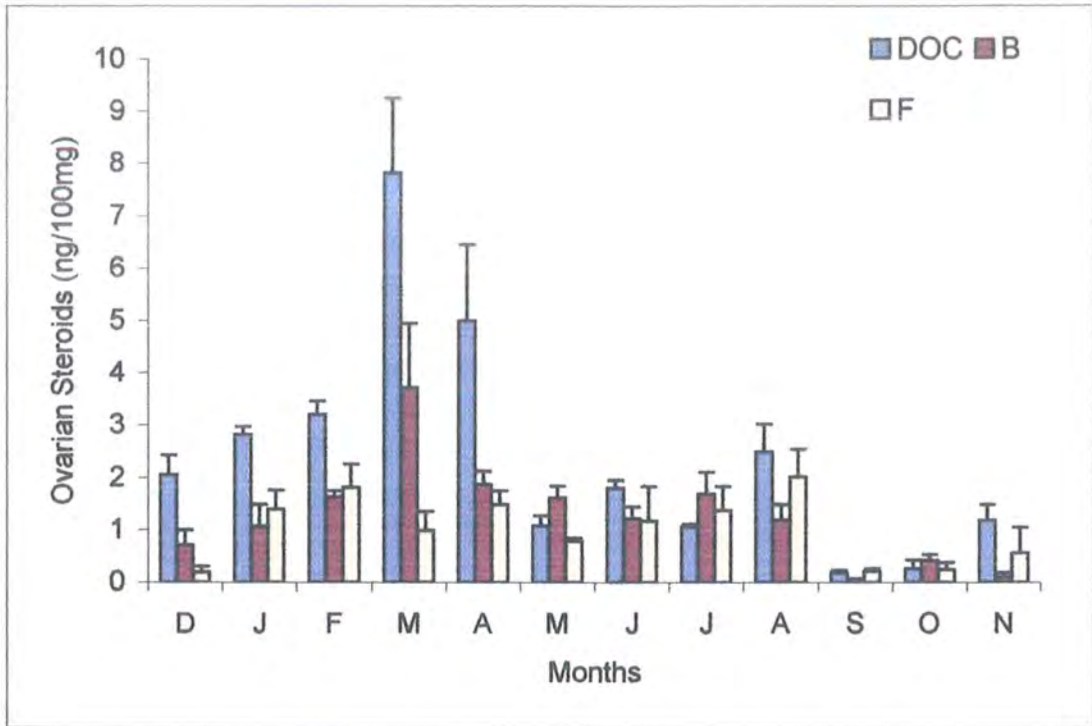

Fig 12. Seasonal variation in ovarian corticosteroids (*Cyprinion watsoni*)

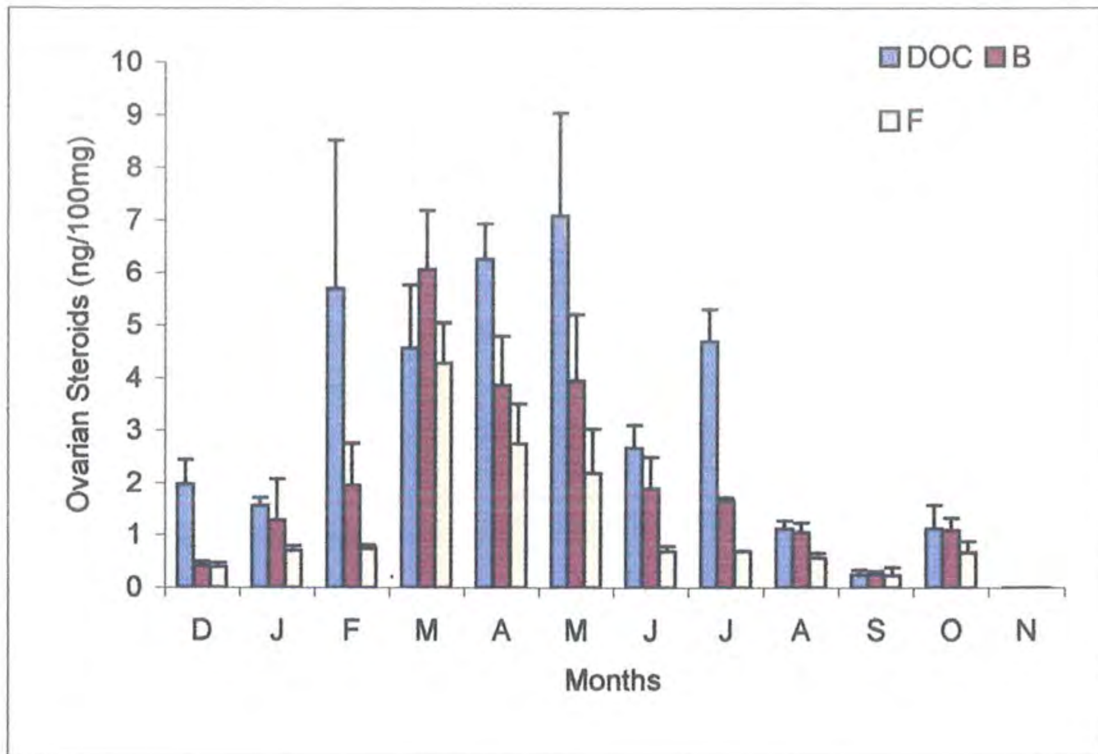

Fig 13. Seasonal variation in ovarian corticosteroids (*Barilius vagra*)

with peaks in March and May. The most conspicuous progestogens in March and April were 17-OHP and 17,20βP, the latter being 3-fold higher than 17-OHP (49 and 14 ng/100 mg, respectively, in March). While a significant decrease of 17-OHP occurred in April, 17, 20βP remained at a high level (> 25 ng/100 mg) in this month before declining further in the succeeding months. Both progestins persisted at levels much higher than in December. The level of both, particularly 17, 20βP, were 2-3 fold higher in *B. vagra* than in *C. watsoni* (Tables 6, 7).

Corticosteroids

Of the corticosteroids, only deoxycorticosterone (DOC), cortisol (F) and corticosterone (B) could be identified in the ovaries of both *C. watsoni* and *B. vagra* (Tables 8, 9; Figs. 12, 13).

In *C. watsoni*, the highest levels were of DOC with peak concentration in March and April (Table 8, Fig. 12). DOC started increasing from lows in the postspawning months (September to November) to a maximum value in March (vitellogenesis) before declining sharply in May. B peaked in March, dropped by nearly 50% in April and prevailed at almost this level until August. F was generally low throughout the year with a marked peak in August. Thus, the dominant steroids in *C. watsoni* were DOC and B.

In *B. vagra*, the level of DOC increased sharply in February, peaked in May and remained quite high even in July (Table 9, Fig. 13). Its second peak in July was coincident with the second GSI peak in this month. The level of B reached a peak during the vitellogenic phase in March (6.0 ng/100 mg), dropping to a little less than 50% of the March value. F also reached its peak concentration in March, decreasing in May to about 50% of the level in March. Its level was extremely low during the rest of the months.

The levels of the three corticosteroids were extremely low in both species in the postspawning months and the level of F was significantly higher in *B. vagra* than in *C. watsoni* in March to May (Figs. 12, 13).

Table 10: Seasonal profile of ovarian aldosterone (ALDO) (ng/100mg ± S.E) in *Cyprinion watsoni*.

	ALDO
December	0.84±0.36
January	1.40±0.27
February	2.83±0.52
March	3.08±0.40
April	3.51±0.66
May	2.60±0.45
June	1.31±0.37
July	0.77±0.40
August	2.54±1.14
September	0.04±0.00
October	0.09±0.02
November	0.45±0.45

Table 11: Seasonal profile of ovarian aldosterone (ALDO) (ng/100mg ± S.E) in *Barilius vagra*.

	ALDO
December	1.48±0.38
January	1.66±0.34
February	2.35±0.22
March	4.30±0.71
April	2.47±0.96
May	2.39±1.02
June	2.51±1.19
July	1.22±0.07
August	1.19±0.18
September	1.54±0.60
October	1.09±0.26
November	n.d

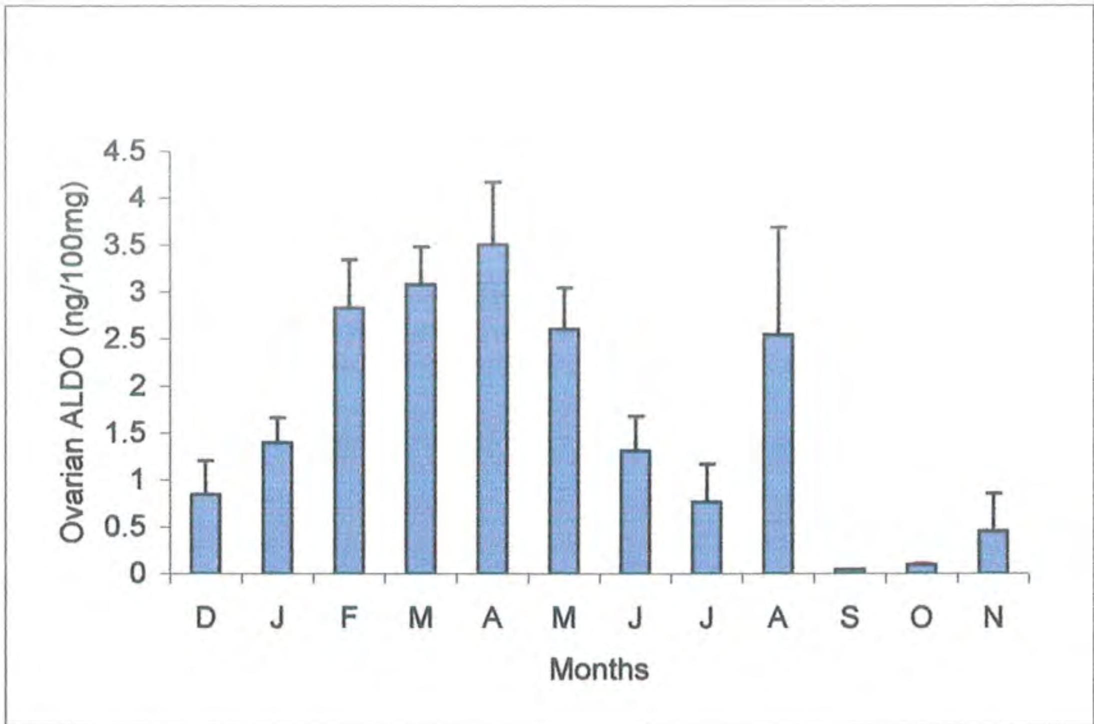

Fig 14. Seasonal changes in ovarian aldosterone (*Cyprinion watsoni*).

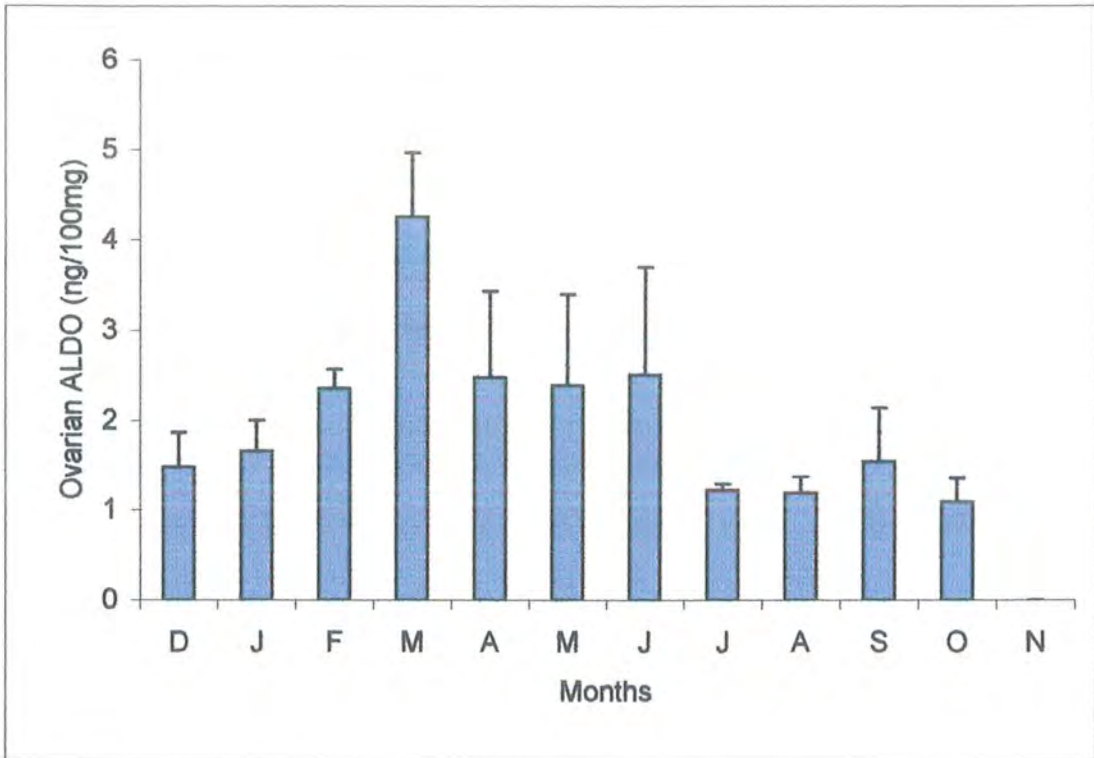

Fig 15. Seasonal changes in ovarian aldosterone (*Barilius vagra).*

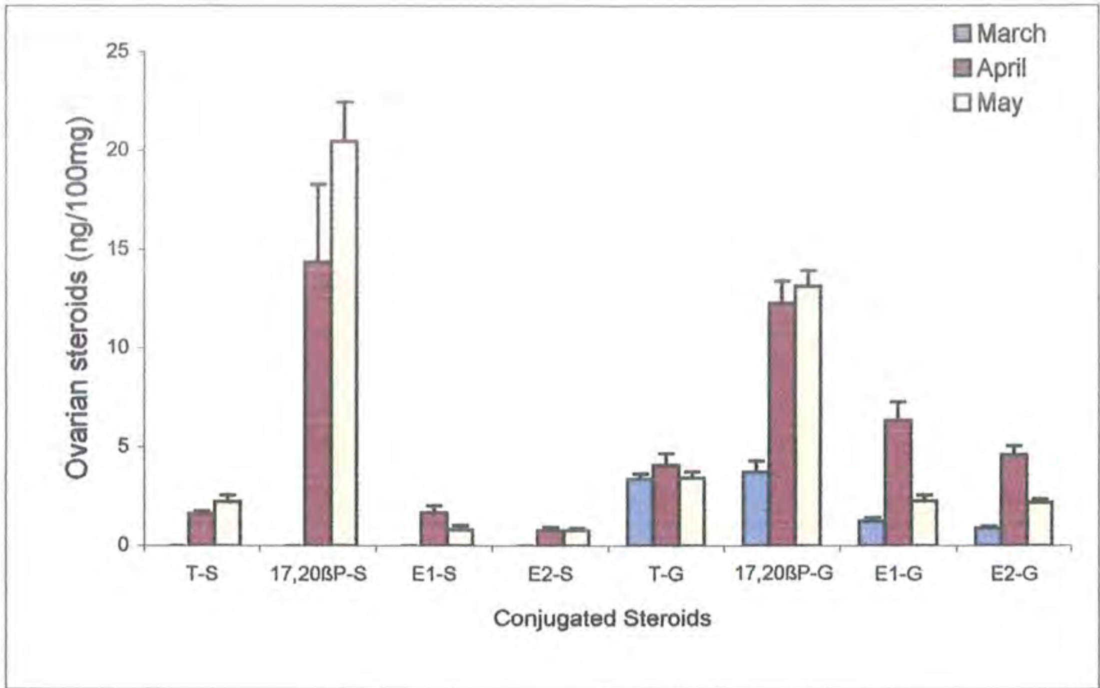

Fig 16. Seasonal changes in conjugated steroids (*Cyprinion watsoni*). *S=sulphate, G= glucuronide.*

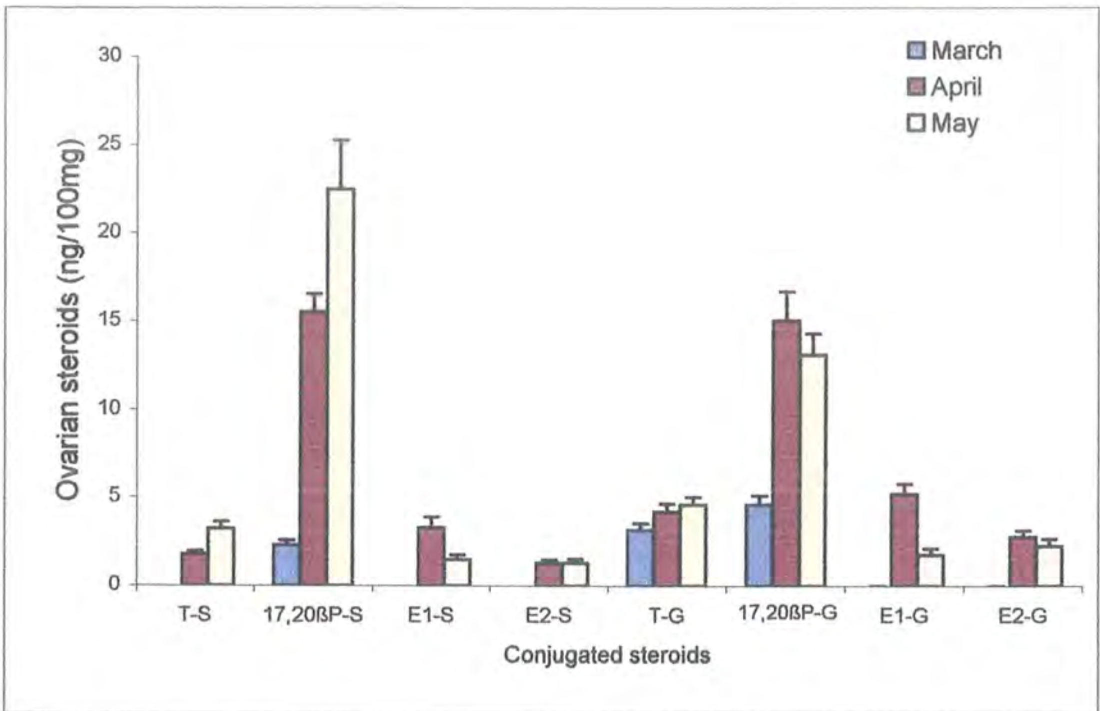

Fig 17. Seasonal changes in conjugated steroids (*Barilius vagra*). *S= sulphate, G=glucuronide.*

Aldosterone

The concentration of aldosterone (ALDO) was lowest in the postspawning months of September to November in *C. watsoni* (Table 10, Fig. 14) and increased sharply to peak levels with vitellogenic progress in February, March and April. It remained at this level until May (around 2.6 ng/100 mg) before declining transiently in June. A second peak occurred in August.

In *B. vagra*, ALDO (Table 11. Fig. 15) reached its peak in March, declined by nearly 50% in April and remained at this level until June. It was below the detection limit in November.

Conjugated Steroids

Conjugated steroids were studied *in vivo* in the two species only in the prespawning and spawning months of March, April and May (Figs. 16, 17). The sulphates of T, 17, 20βP, E_1 and E_2 were present in the *Cyprinion* follicles in only April and May (spawning months), whereas the glucuronide conjugates of these steroids were detected in all three months (Fig. 16). The level of 17, 20βP-S far exceeded the levels of T-S, E_1-S and E_2-S with the onset of spawning (April/May). 17, 20βP-G was also significantly higher than T-G, E_1-G, and E_2-G. Although its concentration in April was comparable to 17, 20βP-S in May, it was markedly lower than its sulphated form in this month. While T-G levels were nearly the same in the three months, those of E_1-G and E_2-G were significantly higher in April (onset of spawning). Of the four glucuronide conjugated steroids, however, 17, 20βP-G was the most pronounced with onset of spawning.

In *B. vagra* too, T-S, E_1-S and E_2-S were undetectable in March (vitellogenesis) (Fig.17). E_1-G and E_2-G were also absent in this month. Significantly higher amounts of 17, 20βP-S prevailed in April and May. 17, 20βP-G also dominated the other glucuronide conjugates with its amount increasing significantly in April/May over the March level. Of the estrogen conjugates, both E_1-G and E_2-G were absent in March but were noticeably high in the spawning period (April-May). Nearly similar levels of

Table 12a: Anova of GVBD at three incubation periods following 15 treatments (*Cyprinion watsoni)*

Source	D.F.	S.S.	M.S.	F	p
Replication	2	176	88	1.0733	n.s
Incubation (I)	2	24941.421	12470.71	152.0964	P<0.001
Treatment (T)	14	81737.826	5838.416	71.207	P<0.001
I X T	28	18586.649	663.809	8.096	P<0.001
Error	88	7215.309	81.992		
Total	134	132657.21			

Table 12b: DMRT of mean GVBD (%) at three incubation periods following 15 treatments (*Cyprinion watsoni*)

Treatments \ Incubation		24 hrs	48 hrs	72 hrs
		13.85^{c}	33.23^{b}	46.99^{a}
Control	2.72^{f}	0.00^{m}	1.97^{lm}	6.17^{ijklm}
E2 (1ug/ml)	4.07^{f}	0.027^{m}	2.84^{klm}	9.34^{ijklm}
E1 (1ug/ml)	3.21^{f}	1.23^{lm}	1.27^{lm}	7.13^{ijklm}
T (1ug/ml)	43.76^{cd}	12.43^{ijklm}	47.20^{fgh}	71.63^{bcd}
AD (1ug/ml)	38.34^{cd}	8.77^{ijklm}	43.83^{h}	62.43^{cdef}
P4 (1ug/ml)	58.32^{b}	19.10^{ijkl}	64.37^{bcde}	91.50^{a}
17-OHP (1ug/ml)	60.32^{b}	21.13^{ij}	66.23^{bcde}	93.60^{a}
17,20ßP (1ug/ml)	76.93^{a}	73.33^{bcd}	91.90^{a}	96.60^{a}
E2 (0.1ug/ml)	3.36^{f}	2.60^{klm}	3.67^{ijklm}	3.80^{ijklm}
E1 (0.1ug/ml)	4.74^{f}	1.07^{lm}	5.56^{jklm}	7.57^{ijklm}
T (0.1ug/ml)	24.70^{e}	4.77^{jklm}	23.86^{i}	45.47^{gh}
AD (0.1ug/ml)	10.37^{f}	1.23^{lm}	9.43^{ijklm}	20.44^{ijk}
P4 (0.1ug/ml)	36.13^{d}	8.63^{ijklm}	38.9^{h}	60.87^{defg}
17-OHP (0.1ug/ml)	46.70^{c}	13.67^{ijklm}	46.27^{fgh}	80.16^{ab}
17,20ßP (0.1ug/ml)	56.72^{b}	39.77^{h}	51.23^{efgh}	79.16^{abc}

All such mean values corresponding to a category (incubations, treatments, I x T) which share a common letter are insignificantly different,otherwise they differ at $p<0.05$.

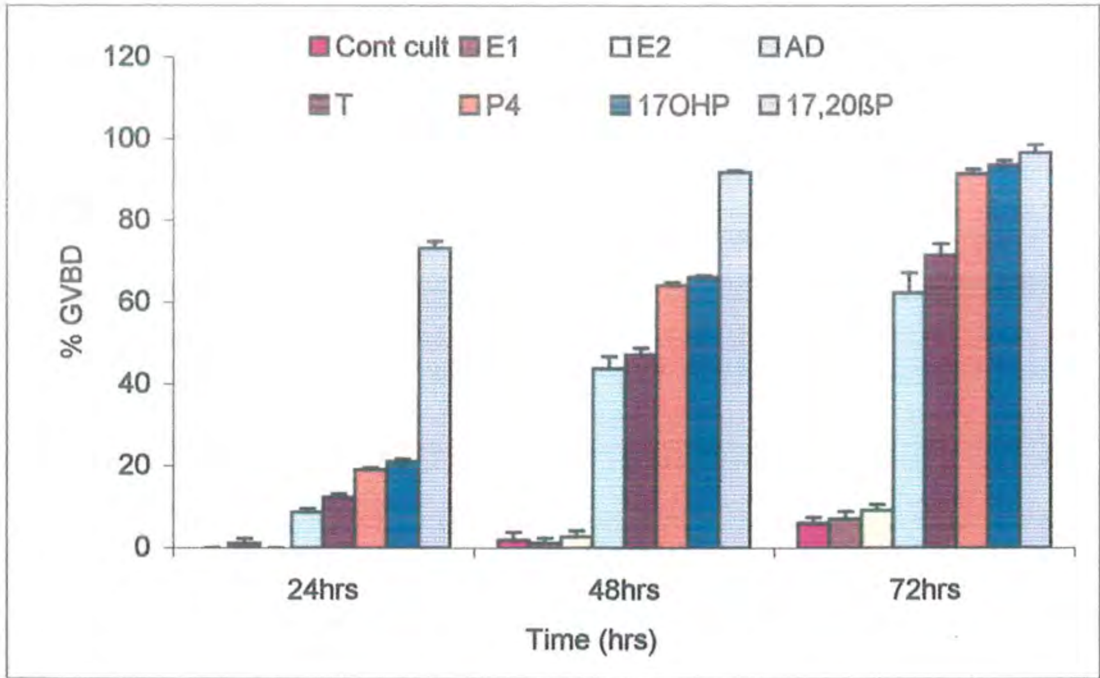

Fig 18. Comparison of *in vitro* induction of germinal vesicle breakdown (GVBD) by different steroids (1ug/ml) without gonadotropin stimulation in oocytes of *Cyprinion watsoni* at three incubation periods.

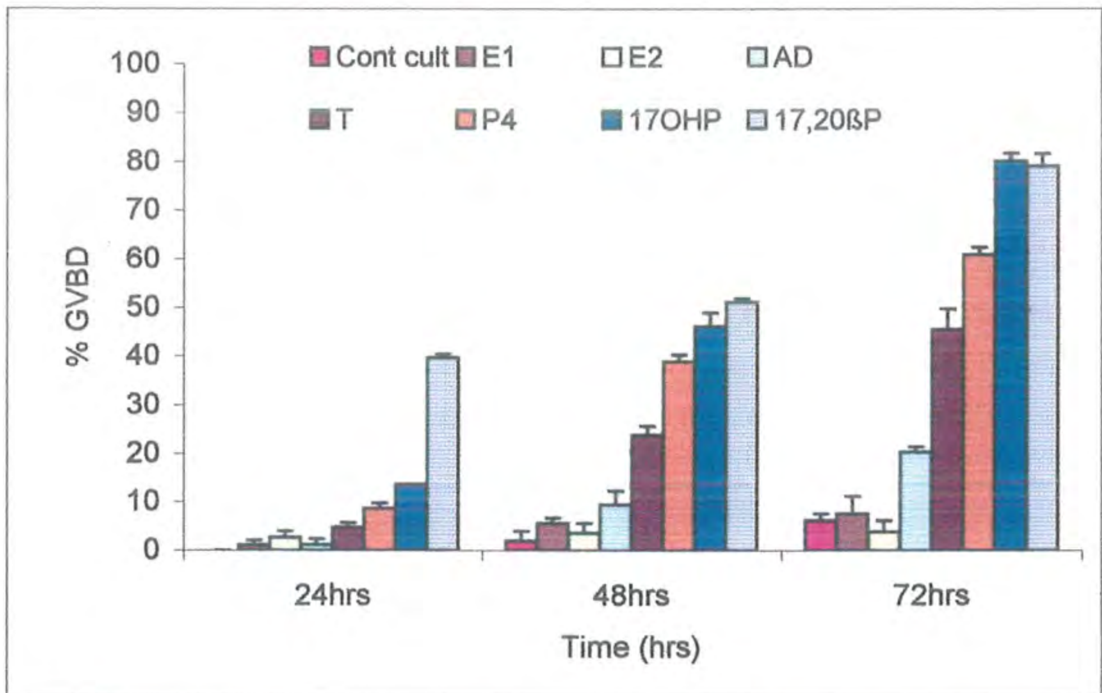

Fig 19. Comparison of *in vitro* induction of germinal vesicle (GVBD) breakdown by different steroids (0.1ug/ml) without gonadotropin stimulation in oocytes of *Cyprinion watsoni* at three incubation periods.

Table13a: Anova of GVBD at three incubation periods following 15 treatments (*Cyprinion watsoni*)

Source	D.F.	S.S.	M.S.	F	p
Replication	2	20.251	10.125	1.0592	n.s
Incubation (I)	2	12850.928	6425.464	672.135	$P<0.001$
Treatment (T)	15	190631.364	12708.758	1329.398	$P<0.001$
I X T	30	9957.121	331.904	34.7188	$P<0.001$
Error	94	898.619	9.56		
Total	143	214358.283			

Table13b: DMRT of mean GVBD (%) at three incubation periods following 15 treatments (*Cyprinion watsoni),* Steroids 0.1ug/ml and hcG 10 IU/ml

Incubation / Treatments		24 hrs	48 hrs	72 hrs
		29.66^{c}	42.96^{b}	52.71^{a}
Control	1.77^{l}	2.57^{pq}	1.50^{q}	1.23^{q}
hcG	7.61^{jk}	3.93^{opq}	5.67^{nopq}	13.23^{lm}
E2 +hcG	13.79^{i}	6.00^{nopq}	10.87^{mn}	24.49^{i}
E1 +hcG	8.94^{i}	3.80^{opq}	6.64^{nopq}	16.37^{kl}
T+hcG	87.64^{c}	71.77^{d}	96.17^{ab}	95.00^{ab}
AD +hcG	81.86^{d}	55.67^{f}	92.20^{b}	97.70^{ab}
P4 +hcG	91.57^{b}	79.67^{c}	97.50^{ab}	97.53^{ab}
17-OHP+hcG	93.29^{b}	81.93^{c}	97.70^{ab}	100.00^{a}
17,20ßP+hcG	99.24^{a}	97.50^{ab}	100.00^{a}	100.00^{a}
E2	3.36^{l}	2.60^{pq}	3.667^{opq}	3.80^{opq}
E1	4.73^{k}	1.07^{q}	5.56^{nopq}	7.57^{nop}
T	24.70^{h}	4.77^{opq}	23.86^{j}	45.47^{h}
AD	10.37^{i}	1.24^{q}	9.433^{mno}	20.44^{jk}
P4	36.13^{g}	8.63^{mno}	38.90^{i}	60.87^{e}
17-OHP	46.70^{f}	13.67^{lm}	46.27^{gh}	80.16^{c}
17,20ßP	56.72^{e}	39.77^{i}	51.23^{fg}	79.16^{c}

All such mean values corresponding to a category (incubations, treatments, I x T) which share a common letter are insignificantly different,otherwise they differ at $p<0.05$.

T-G prevailed in March, April and May but tended to gradually increase as May approached (spawning period).

IN VITRO INDUCTION OF OOCYTE MATURATION BY STEROIDS

Since it has been shown in other studies (Van Ree *et al.*, 1977; Pankhurst, 1985) that oocytes of various species of fish are capable of undergoing spontaneous GVBD, fresh samples of intrafollicular oocytes were subjected to preliminary examination of initial position of the GV and the extent of GVBD, if any, allowing comparisons with the extent of maturational change (GVBD) in the control incubations (untreated). Over 90% of the oocytes had central GV and none showed GVBD, at least until 48 hr of incubation in the untreated medium as was the case in the initial (fresh unincubated) samples of the ovary. Thus, exposure of the oocytes to the culture medium alone had no influence on the GV, at least until 72 hr when at most ~ 6% oocytes showed GVBD in *C. watsoni* (Tables 12b, 13b, 14b) and <13% showed GVBD in *B. vagra* (Table 15b).

Seven steroids, namely, AD, T, E_1, E_2, P_4, 17-OHP and 17, 20βP, individually and in combination with hCG (10 IU/ml), were selected for *in vitro* induction of oocyte maturation (GVBD) at 3 different incubations (24, 48, 72 hr) at various dosage levels in triplicate. The Anova and Duncan's Multiple Range Test (DMRT) analysis of the data (GVBD; control and test cultures) are shown in Tables 12 to 14 (*C. watsoni*) and in Table 15 (*B. vagra*). The results of Anova indicate that there was no statistically significant difference amongst the three replicates of each treatment (i.e. the results were reproducible) but the differences amongst the three incubation periods (24, 48 and 72 hr) as well as amongst the hormonal treatments were highly significant ($P< 0.001$). Thus, both the duration of exposure and the hormonal treatments had a positive influence on the behavior of GV.

Cyprinion watsoni: *in vitro* induction of maturation

Comparison of the changes in mean GVBD in the control and treated incubations according to Duncan's Multiple Range Test are shown in Tables 12b to 14b (Figs. 18, 19). The top row and the first column in the Tables show the mean GVBD percentage

Table 14 a. Anova of in vitro induction of germinal vesicle breakdown (GVBD) in *Cyprinion watsoni*: Comparision of potency of 15 different treatments

Source	D.F.	S.S.	M.S.	F	p
Replication	2	7.984	3.992	0.2335	n.s
Treatment (T)	15	54840.1	3656	213.82	P<0.001
Error	30	512.963	17.099		
Total	47	55361.1			

Table 14b. DMRT of mean in vitro induction of germinal vesicle breakdown (GVBD %) in *Cyprinion watsoni*: Comparision of potency at three different concentrations of different hormones.

Treatments	%GVBD
Control culture	6.17^{i}
T (1ug/ml)	71.63^{d}
AD (1ug/ml)	62.43^{e}
P4 (1ug/ml)	91.50^{b}
17-OHP (1ug/ml)	93.60^{ab}
17,20ßP (1ug/ml)	98.90^{a}
T (0.1ug/ml)	45.47^{f}
AD (0.1ug/ml)	20.44^{g}
P4 (0.1ug/ml)	60.87^{e}
17-OHP (0.1ug/ml)	80.16^{c}
17,20ßP (0.1ug/ml)	79.16^{c}
T (0.01ug/ml)	5.52^{i}
AD (0.01ug/ml)	5.20^{i}
P4 (0.01ug/ml)	14.47^{gh}
17-OHP (0.01ug/ml)	8.74^{hi}
17,20ßP (0.01ug/ml)	49.87^{f}

All such mean values corresponding to a category (incubations, treatments, I x T) which share a common letter are insignificantly different,otherwise they differ at $p<0.05$.

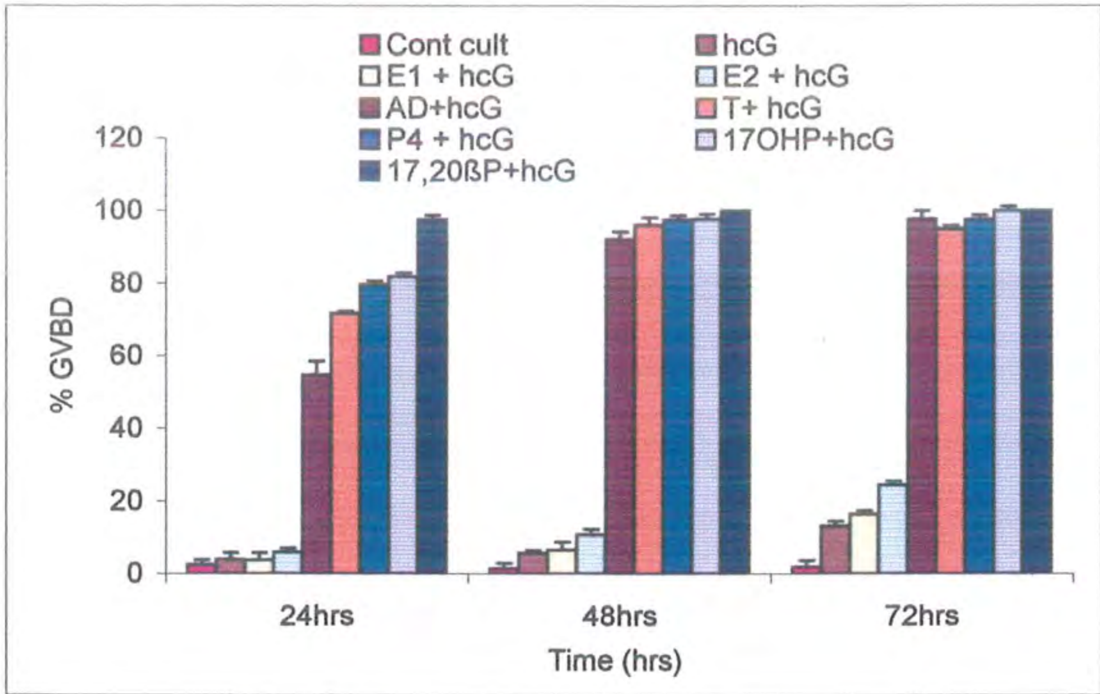

Fig 20. Comparison of *in vitro* induction of germinal vesicle breakdown (GVBD) by different steroid hormones (0.1ug/ml) in combination with hcG (10 IU/ml) in oocytes of *Cyprinion watsoni* at three incubation periods.

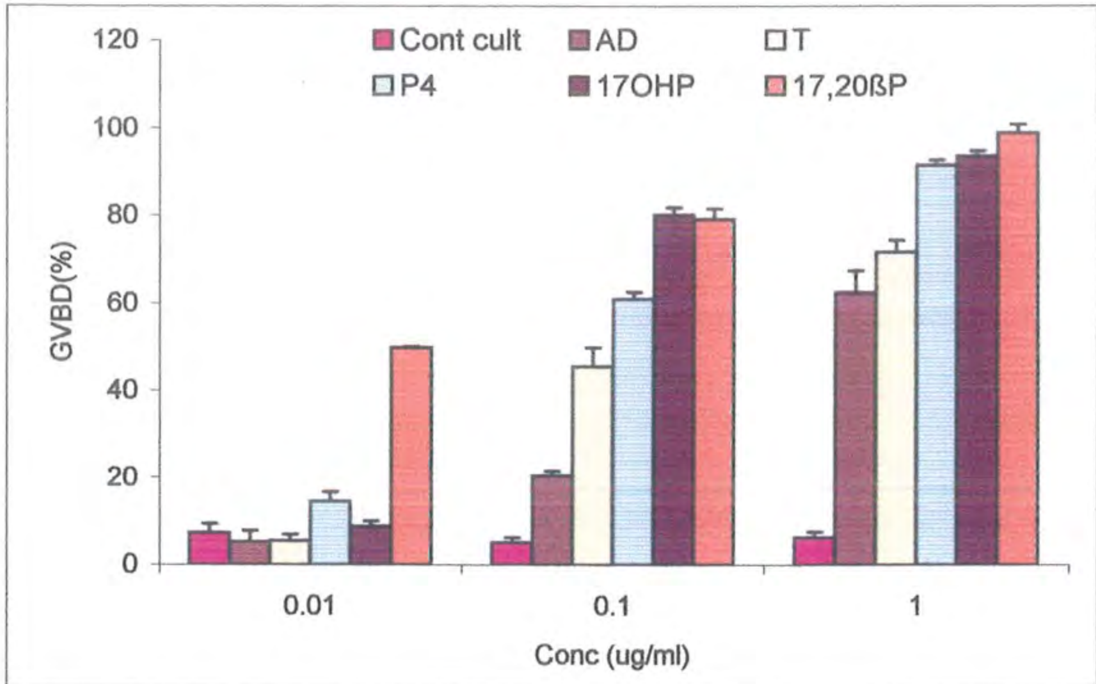

Fig 21. *In vitro* Induction of germinal vesicle breakdown (GVBD) in oocytes of *Cyprinion watsoni* by selected androgens and progestrogens at three different concentrations following 72hrs incubation.

and significance of differences (at $P < 0.05$) amongst the incubations and the treatments). The three incubations differed significantly ($P < 0.05$). The treatments (estrogens, androgens and progestins) also showed significant differences ($P < 0.05$) in mean GVBD percentage. While the estrogens and the control treatment were insignificantly different, the androgens and the progestins caused significantly greater GVBD compared to the former treatments, excepting AD at 1.0 ug/ml. At both concentrations (1.0 and 0.1 ug/ml), only 17, 20βP had the most significant GVBD effect (Table 12b). Finally, whereas all androgens and progestins caused significant GVBD at 1.0 ug/ml, only T and the progestins had a significant GVBD effect at 0.1 ug/ml. When interaction amongst the individual treatments and the incubation times (I x T) is taken into account, it is evident that percentage GVBD increased significantly ($P < 0.05$) at both concentrations (1.0 and 0.1 ug/ml) at 48 hr and still further at 72 hr on exposure to only the androgens and the progestogens. At 1.0 ug/ml, the three progestogens had a comparable and very high GVBD %age at 72 hr. It decreased noticeably at 0.1 ug/ml but only 17-OHP and 17, 20βP had the most outstanding influence (~80% GVBD) compared to P_4 and the androgens; the sequence being 17, 20βP > 17-OHP > P_4 > T. E_1 and E_2 were entirely ineffective in causing significant GVBD at all incubation intervals and at both concentrations (Figs. 18, 19).

Table 13 (Fig. 20) shows the effect of the various exogenous steroids alone and in combination with hCG along with the control (untreated). Again, the effect of incubations was significant ($P < 0.05$; top row, Table 13b). hCG alone had a slight but significant GVBD effect compared to the control. It also significantly promoted the GVBD response to all steroids applied; the most significant and dramatic increases occurring with only the androgens and the progestins (17, 20βP > 17-OHP/P_4 > T > AD). Maximum and the most significant effect was that of 17, 20βP + hCG (~99% GVBD). The GVBD response to 17, 20βP + hCG was significantly greater compared to all other steroids. In terms of interaction (I x T), the promotional effect of hCG on all steroids increased as the incubation intervals changed from 24 to 72 hr (Table 13b, horizontal rows). The most profound effect already at 48 hr occurred with the androgens and the progestogens (92-100% GVBD). At 72 hr, 100% GVBD was caused by both 17-OHP and 17, 20βBP in

Table 15a: Anova of GVBD at three incubation periods following 13 treatments *(Barilius vagra)*

Source	D.F.	S.S.	M.S.	F	p
Replication	2	8.376	4.188	0.9236	n.s
Incubation (I)	2	16123.787	8061.894	1778.064	P<0.001
Treatment (T)	12	103353.91	8612.826	1899.573	P<0.001
I X T	24	6416.94	267.372	58.9694	P<0.001
Error	76	344.59	4.534		
Total	116	126247.6			

Table 15b: DMRT of mean GVBD (%) at three incubation periods following 13 treatments *(Barilius vagra)*. Steroids 1ug/ml and hcG 1.0 IU/ml

Treatments \ Incubation		24 hrs	48 hrs	72 hrs
		31.03^{c}	48.75^{b}	59.5^{a}
Control culture	7.71^{j}	2.90^{wx}	7.35^{uv}	12.87^{rst}
E1	8.38^{j}	1.24^{x}	6.30^{vw}	17.62^{pq}
E2	10.53^{i}	5.05^{vw}	10.78^{tu}	15.77^{qrs}
AD	24.49^{g}	12.47^{st}	21.90^{o}	39.10^{l}
T	48.92^{f}	20.7^{op}	58.43^{i}	67.62^{h}
P4	47.19^{f}	22.38^{no}	53.13^{i}	66.07^{h}
17-OHP	56.67^{e}	28.00^{m}	64.23^{h}	77.77^{ef}
17,20ßP	89.11^{b}	74.47^{fg}	92.80^{b}	100^{a}
DOC	48.64^{f}	16.71^{q}	48.17^{k}	81.03^{de}
hCG	17.44^{h}	10.23^{tu}	16.43^{qr}	25.67^{mn}
P4 +hcG	68.82^{d}	50.00^{jk}	74.00^{g}	82.47^{d}
17-OHP+hcG	82.47^{c}	76.30^{fg}	83.60^{d}	87.50^{c}
17,20ßP+hcG	93.21^{a}	82.97^{d}	96.66^{a}	100^{a}

All such mean values corresponding to a category (incubations, treatments, I x T) which share a common letter are insignificantly different,otherwise they differ at $p<0.05$.

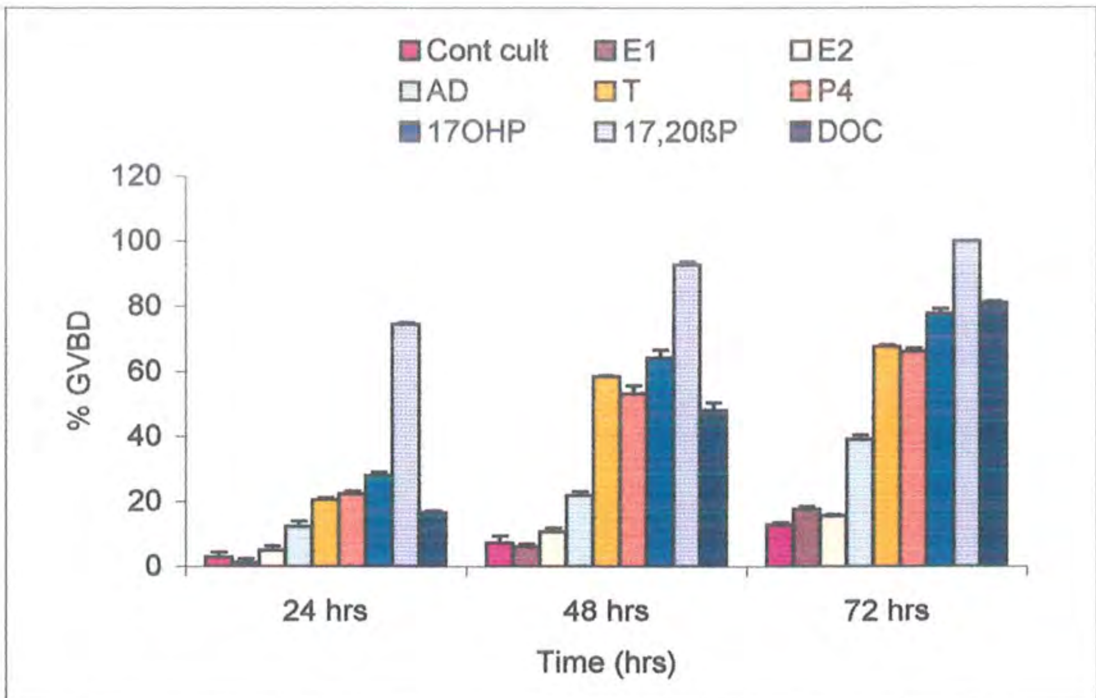

Fig 22. Comparison of *in vitro* induction of germinal vesicle breakdown (GVBD) by different steroid hormones in oocytes of *Barilius vagra* at three incubation periods.

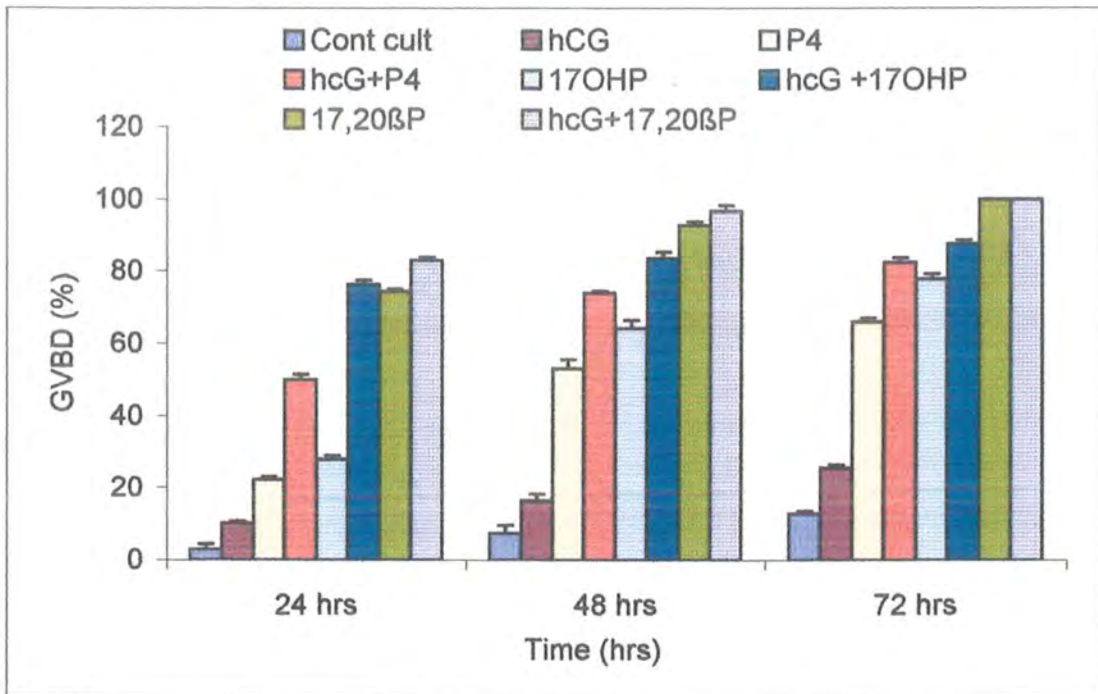

Fig 23. Comparison of *in vitro* induction of germinal vesicle breakdown (GVBD) by hCG alone, progestogens alone and in combination with hCG (10 IU/ml) in oocytes of *Barilius vagra* at three incubation periods.

combination with hCG. Without hCG, only 17-OHP and 17, 20βP had the most pronounced and nearly comparable effect at 72 hr (~80% GVBD, Table 13b). The least priming effect of hCG evidently was on the estrogens.

In view of the efficiency of the androgens and progestogens in influencing maturation (GVBD), their effects were further tested at concentrations of 1.0, 0.1 and 0.01 ug/ml using only 72 hr incubations (Table 14, Fig. 21). Although the androgens and the progestogens had a significant effect at 1.0 and 0.1 ug/ml compared to the control, only 17,20βP was singularly effective in causing significant GVBD (50% GVBD) at a concentration of 0.01 ug/ml at 0.1 ug/ml. Also, only the progestogens had by far the greatest and similar effect, 17-OHP and 17, 20βP being similar in terms of GVBD response. From the results, it is evident that reduction in dosage (to what may be near physiological levels) caused a decrease in response to the various progestogens (Table 14b, Fig. 21).

Barilius vagra: *in vitro induction of maturation*

The Anova and DMRT of mean central position of GV and mean GVBD following treatment with steroids alone (1.0 ug/ml) and with hCG (1 IU/ml) for *B. vagra* are shown in Table 15 (Figs. 22, 23). In the control no more than ~13% follicles showed GVBD.

The response at the three incubations differed significantly ($P < 0.05$). Regarding the effect of the estrogen treatments, only E_2 differed significantly from the control. While all of the androgens and progestins caused significant GVBD, the effect of both 17-OHP and 17, 20βP was much greater. However, 17, 20βP was the single outstanding steroid causing 89% GVBD. Interestingly, DOC also had an effect comparable to that of T and P_4. Taking I x T into account, a significant but marginal increase in GVBD occurred on a time-dependent basis with the estrogens. Such increase was far greater following incubation with the androgens and the progestogens, 17, 20βP causing 100% GVBD at 72 hr (Table 15b). The effect of hCG alone differed significantly from the control (17.4% vs 7.7%, Table 15b, Fig. 23). With prolonged incubation further increase

Table 16: Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* with and without hcG (10 IU/ml) for 72hrs. S=sulphate, G= glucuronide.

	Control			hcG treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	–	–	–
F	0.55±0.20	–	–	–	–	–
B	1.18 ±0.23	–	–	2.13±0.41	–	–
DOC	1.01±0.24	–	–	1.28±0.32	–	–
19OHA	1.13±0.18	–	–	–	–	–
11ßOHA	0.73±0.28	–	–	1.88±0.49	–	–
AD	1.30±0.32	–	–	2.90±0.25	–	–
T	1.23±0.26	–	–	3.86±0.38	–	0.73±0.42
17-OHP	–	–	–	1.23±0.32	–	–
17,20ßP	–	–	–	0.97±0.20	–	–
P4	1.70±0.26	–	–	3.44±0.37	–	–
E	0.16±0.04	–	–	3.46±0.66	–	–
E1	2.30±0.43	–	–	5.10±0.92	3.2±0.77	–
E2	1.35±0.29	–	–	6.60±1.24	1.70±0.35	4.40±0.61

occurred (26% at 72 hr). The gonadotropin also promoted the GVBD response to all progestins, especially 17, 20βP (100% GVBD, Table 15b, Fig. 23). The relative response was 17, 20βP > 17-OHP > P_4.

RECOVERY OF STEROID METABOLITES FROM THE INCUBATION MEDIUM (*C. watsoni*, 72 hr incubation)

Control incubation

Table 16 shows levels of free steroid metabolites recovered from the medium by incubating the intrafollicular oocytes of *C. watsoni* for 72 hr. The free estrogens and androgens as well as P_4, ALDO and corticosteroids were recovered from the incubation medium. Of the estrogens and androgens, the highest quantity recovered was that of E_1 and the least was that of E. The levels of E_2, T, AD, 19-OHA, 11β-OHA, P_4, F, B and DOC were statistically comparable. The quantities of ALDO and E_1 were nearly similar, above 2.0 ng/ml. Surprisingly, both free 17-OHP and 17, 20βP were absent. Conjugated steroids were also not detectable.

hCG-treated follicles

When the follicles were exposed to hCG (10 IU/ml), ALDO, F and 19-OHA were undetectable in the incubation medium, suggesting apparent inhibition of these steroids (Table 16). The levels of B, DOC, the remaining androgens, estrogens and progestogens were enhanced by hCG. The levels of E, E_1 and particularly of E_2 were maximally enhanced over and above the control values under the influence of hCG. Notable increases over the control levels were also recorded in the quantities of 11β-OHA, T, AD and P_4. While 17-OHP and 17, 20βP were below the detection limit in the control incubation, these metabolites, particularly 17-OHP appeared in the medium. Maximum levels, however, were those of the free E_2. Conjugated metabolites recovered from the medium were only those of T (glucuronide) and the three estrogens (Table 16). While E_1 appeared in only sulphated form, E_2 appeared in both its sulphated and glucuronide forms, of which the maximum quantity was that of E_2-G.

Table 17. Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* with and without estrone (0.1ug/ml) for 72hrs. S=sulphate, G= glucuronide.

	Control			E1 treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	2.96± 0.63	–	–
F	0.55±0.20	–	–	–	–	–
B	1.18 ±0.23	–	–	1.56±0.20	–	–
DOC	1.01±0.24	–	–	0.73±0.22	–	–
19OHA	1.13±0.18	–	–	–	–	–
11ßOHA	0.73±0.28	–	–	0.92±0.31	–	–
AD	1.30±0.32	–	–	2.20±0.35	–	–
T	1.23±0.26	–	–	1.46±0.84	–	–
17-OHP	–	–	–	0.67±0.17	–	–
17,20ßP	–	–	–	–	–	–
P4	1.70±0.26	–	–	1.42±0.26	–	–
E	0.16±0.04	–	–	3.40±0.49	–	–
E1	2.30±0.43	–	–	52.8±2.45	12.20±0.80	9.10±0.46
E2	1.35±0.29	–	–	7.80±0.98	1.58±0.23	2.46±0.34
UKN 20.9	–	–	–	+	–	–

Table 18. Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* with and without estradiol (0.1ug/ml) for 72hrs. S=sulphate, G= glucuronide.

	Control			E2 treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	2.70±0.3	–	–
F	0.55±0.20	–	–	–	–	–
B	1.18 ±0.23	–	–	1.73±0.27	–	–
DOC	1.01±0.24	–	–	0.69±0.09	–	–
19OHA	1.13±0.18	–	–	–	–	–
11ßOHA	0.73±0.28	–	–	0.83±0.19	–	–
AD	1.30±0.32	–	–	2.00±0.30	–	–
T	1.23±0.26	–	–	0.66±0.13	–	–
17-OHP	–	–	–	0.67±0.12	–	–
17,20ßP	–	–	–	–	–	–
P4	1.70±0.26	–	–	1.29±0.2	–	–
E	0.16±0.04	–	–	4.66±0.93	–	–
E1	2.30±0.43	–	–	39.90±1.06	8.20±0.93	2.37±0.32
E2	1.35±0.29	–	–	17.80±1.0	6.00±0.79	10.5±1.27
UKN 29.5	–	–	–	+	–	–

Estrogen-treated follicles

Table 17 shows recovery of the free steroid metabolites from the control (untreated) and E_1-treated media. All free steroid metabolites were recovered from the control incubation. 17-OHP and E_1 were close to the undetectable limit in the control incubation. In the E_1-treated incubation, ALDO, F, 19-OHA and 17, 20βP were not detectable. The quantities of DOC, 11β-OHA and 17-OHP were quite low. Only about 52% E_1 substrate was recovered as such. Significantly high quantities of E_2 and E were recovered from the treated incubation, indicating appreciable conversion of E_1 into the former steroids. The levels of the androgens and P_4 recovered from the incubation were comparable to the recovery from the control incubation. The most significant conversions of E_1 were into conjugated steroids (Table 17). A major portion of this estrogen appeared as E_1-S and G. The quantities of E_2-S and G were substantially lower. A peak of an unknown free steroid was also detectable.

Table 18 shows recovery of various steroids from the control and E_2-treated incubations. In the E_2-treated incubation, only about 18% of the substrate was recovered as such, while the quantity of free E_1 was around 40% and ~ 5% that of E, suggesting substantial metabolization of E_2 by the follicles. F, 19-OHA and 17, 20βP were undetectable, while the levels of the other steroids were comparable to their levels in the control incubation. An unidentifiable peak of free steroid was also recorded in the E_2-treated incubations. In addition, a substantial quantity of E_2 was converted into conjugated E_1 and E_2, both sulphate and glucuronide forms (Table 18). The dominant quantities were those of E_1-S and E_2-G.

Androgen-treated follicles

In the testosterone-treated incubation, significant quantity of T was converted into AD, E_1 and E_2, and only about 8% of the T substrate was recovered as such (Table 19). Of these metabolites, the estrogens (E_1 and E_2) were dominant. The quantities of the other free metabolites were nearly comparable to the levels in the control incubation (~ 2ng/ml or less in the control). 17-20βP was below the detectable limit. T-G, E_1-S as well as E_2-S and G conjugates were also recovered from the incubation (Table 19). Three peaks of

Table 19. Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* with and without testosterone (0.1ug/ml) for 72hrs. S=sulphate, G= glucuronide.

	Control			T treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	–	–	–
F	0.55±0.20	–	–	0.55±0.06	–	–
B	1.18 ±0.23	–	–	0.86±0.07	–	–
DOC	1.01±0.24	–	–	1.21±0.31	–	–
19OHA	1.13±0.18	–	–	1.00±0.20	–	–
11ßOHA	0.73±0.28	–	–	1.56±0.06	–	–
AD	1.30±0.32	–	–	34.65±2.27	–	–
T	1.23±0.26	–	–	8.28±0.55	–	4.60±0.40
17-OHP	–	–	–	2.85±0.14	–	–
17,20ßP	–	–	–	–	–	–
P4	1.70±0.26	–	–	1.56±0.32	–	–
E	0.16±0.04	–	–	2.50±0.20	–	–
E1	2.30±0.43	–	–	14.53±1.22	2.47±0.18	–
E2	1.35±0.29	–	–	13.43±0.50	2.10±0.17	2.33±0.15
UNK30.8	–	–	–	+	+	–
UNK42.5	–	–	–	+	+	–
UNK46.5	–	–	–	+	–	–

Table 20. Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* with and without androstenedione (0.1ug/ml) for 72hrs. S=sulphate, G= glucuronide.

	Control			AD treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	–	–	–
F	0.55±0.20	–	–	1.01±0.15	–	–
B	1.18 ±0.23	–	–	1.14±0.17	–	–
DOC	1.01±0.24	–	–	1.57±0.20	–	–
19OHA	1.13±0.18	–	–	3.48±0.38	–	–
11ßOHA	0.73±0.28	–	–	2.66±0.34	–	–
AD	1.30±0.32	–	–	34.33±1.78	–	–
T	1.23±0.26	–	–	3.63±0.55	–	5.63±0.71
17-OHP	–	–	–	–	–	–
17,20ßP	–	–	–	2.80±0.23	–	–
P4	1.70±0.26	–	–	1.17±0.23	–	–
E	0.16±0.04	–	–	–	–	–
E1	2.30±0.43	–	–	10.6±1.07	–	1.57±0.39
E2	1.35±0.29	–	–	2.54±0.49	–	–
UNK30.8	–	–	–	+	+	–
UNK42.5	–	–	–	+	–	–
UNK 40.5	–	–	–	+	–	–

both free and conjugated steroid metabolites that could not be identified were also detected (Table 19).

When the follicles were incubated with AD, significantly high quantity of free E_1 was recovered from the incubation and ~34% of the substrate was recovered as such (Table 20). The next significant quantities were those of T, 19-OHA, followed by 17-20βP, E_2 and 11β-OHA. ALDO did not appear in the medium. The quantities of the other free steroid metabolites were comparable to those in the control incubation, while 17-OHP and P_4 were below the detectable limit. Three peaks of free steroids and one of a conjugated steroid that could not be identified were also detected (Table 20). The conjugated steroids recovered were only glucuronides of T and E_1, of which T-G was significantly higher.

Progestogen-treated follicles

Table 21 lists steroid metabolites following incubation of the follicles with progesterone and in the control incubation medium. A substantial quantity of the substrate was mobilized along various pathways. Compared to the control, significantly higher levels of T, 11β-OHA, E_1, E_2, DOC, B, 17-OHP and 17, 20βP were recovered from the treated incubation. Of these, the most dominant steroids were T, DOC and 17-OHP. Only ~27% of the free substrate was recovered as such. ALDO, F and 19-OHA were undetectable. Three peaks of unidentifiable free steroids and one of sulphated conjugate were also detected (Table 21). 11β-OHA and 17, 20βP were recorded in both sulphate and glucuronide form. Of these, the latter was significantly higher. In addition, the conjugates of both B and T occurred only in glucuronide form.

When progesterone (P_4) was added in combination with hCG (10 IU/ml), only ~4% of the P_4 was recovered as such (Table 21). Significantly high quantities of free 17-20βP and 17-OHP were recovered from the medium. ALDO, F, 19-OHA, E and E_1 could not be detected. Whereas the levels of the other free steroids were quite similar to their levels in the control incubation, several conjugated steroids appeared in the medium. The highest quantity was that of 17, 20βP-S followed by 17, 20βP-G, T-G, 11β-OHA and AD-G in that order (Table 21), suggesting that hCG promoted substantial metabolization of P_4 along various pathways.

Table 21. Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* in the presence of progesterone alone (0.1ug/ml) and with hcG (10 IU/ml) for 72hrs. S=sulphate, G= glucuronide.

	Control			P4 treated			P4 + hcG treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	–	–	–	–	–	–
F	0.55±0.20	–	–	–	–	–	–	–	–
B	1.18 ±0.23	–	–	4.50±0.42	–	2.97±0.23	2.37±0.32	–	–
DOC	1.01±0.24	–	–	6.17±0.38	–	–	1.57±0.19	–	–
19OHA	1.13±0.18	–	–	–	–	–	–	–	–
11ßOHA	0.73±0.28	–	–	4.70±0.42	2.50±0.35	2.67±0.22	3.26±0.35	4.80±0.29	–
AD	1.30±0.32	–	–	2.27±0.20	–	–	1.03±0.26	–	1.71±0.22
T	1.23±0.26	–	–	7.75±0.64	–	2.48±0.54	1.43±0.27	–	4.80±0.44
17-OHP	–	–	–	5.87±0.96	–	–	16.50±0.80	–	–
17,20ßP	–	–	–	4.07±0.47	4.77±0.49	1.78±0.32	17.37±0.43	16.70±0.32	5.17±0.47
P4	1.70±0.26	–	–	27.47±1.76	–	–	3.63±0.35	–	–
E	0.16±0.04	–	–	2.30±0.32	–	–	–	–	–
E1	2.30±0.43	–	–	4.47±0.44	–	–	–	–	–
E2	1.35±0.29	–	–	4.93±0.38	–	–	–	–	–
UNK19.1	–	–	–	+	–	–	+	+	–
UNK30.8	–	–	–	+	+	–	+	+	+
UNK42.5	–	–	–	+	–	–	+	–	–

When the follicles were incubated with 17-OHP, a substantial amount of it was metabolized to form free as well as sulphated and glucuronidated 17, 20βP followed by 11β-OHA, DOC, B, AD and E_2 (Table 22). About 37% of the 17-OHP substrate was recovered as such. Three unknown peaks of free steroids and two of sulphate conjugates also appeared in the medium (Table 22). Treatment of the follicles with 17-OHP in the presence of hCG (10 IU/ml) resulted in appreciable conversion of this substrate into free 17-20βP and much greater mobilization in the direction of conjugated forms of 17-20βP. 17-20βP-S was almost 3-fold higher than its free form and almost 4-fold higher than its glucuronate conjugate (Table 22). Only ~10% of the 17-OHP substrate was recovered as such, while ALDO, F, 19-OHA, E, E_1 and E_2 were not detectable. The other free steroids were nearly comparable to their quantities in the control medium. Three peaks of unidentifiable free steroids and two of unknown sulphate conjugates were also recorded (Table 22).

A large quantity (32.5%) of conjugated 17, 20βP was recovered following incubation with 17, 20βP (Table 23). An amount of the substrate nearly equal to that of 17, 20β-S was recovered as such. The levels of 11β-OHA, B, AD, 17-OHP and T were either lower than or comparable to their levels in the control incubation. ALDO, F, E, E_1 and E_2 were undetectable. Interestingly, a large number of peaks of free steroids that could not be identified due to lack of the desired standards were also detected (Table 23). Incubation of the follicles with hCG + 17, 20βP resulted in significantly high production of 17, 20βP-S followed by its glucuronide form (Table 23). Only about 17% of the progestogen substrate was recovered as such. The quantity of 17-20βP-G was 1/3 of its sulphate form. The only other identifiable free steroid was AD but its quantity was much less than that recorded for the control incubation. It is evident that hCG apparently inhibited production of most of the androgens, estrogens and corticoids. Several other peaks were detected, the identity of which could not be ascertained. These belonged to both the free and conjugated forms (Table 23).

Table 22. Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* in the presence of 17-OHP (0.1ug/ml) with and without hcG (10 IU/ml) stimulation for 72hrs. S=sulphate, G= glucuronide.

	Control			17-OHP treated			hcG+17-OHP treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	–	–	–	–	–	–
F	0.55±0.20	–	–	–	–	–	–	–	–
B	1.18 ±0.23	–	–	5.13±0.41	–	–	1.20±0.16	–	–
DOC	1.01±0.24	–	–	5.6±0.40	–	–	2.13±0.35	–	–
19OHA	1.13±0.18	–	–	–	–	–	1.18±0.17	–	–
11ßOHA	0.73±0.28	–	–	7.57±0.66	–	–		–	–
AD	1.30±0.32	–	–	3.58±0.25	–	–	2.39±0.29	–	–
T	1.23±0.26	–	–	2.23±0.30	–	–	1.51±0.21	–	–
17-OHP	–	–	–	36.87±1.21	–	–	10.4±0.83	–	–
17,20ßP	–	–	–	17.17±1.33	17.17±0.28	4.2±0.4	12.53±0.71	32.40±2.18	8.37±0.92
P4	1.70±0.26	–	–	1.93±0.20	–	–	+	–	–
E	0.16±0.04	–	–	–	–	–	–	–	–
E1	2.30±0.43	–	–	–	–	–	–	–	–
E2	1.35±0.29	–	–	3.17±0.23	+	–	–	–	–
UNK19.1	–	–	–	+	+	–	–	–	–
UNK24.5	–	–	–	–	–	–	+	++	–
UNK30.8	–	–	–	+	–	–	–	–	–
UNK42.5	–	–	–	+	–	–	+	–	–
UNK46.5	–	–	–	–	–	–	+	++	–

Table 23. Quantities (mean ± S.E) of free (F) and conjugated steroid metabolites produced by incubating oocytes of *Cyprinion watsoni* in the presence of 17,20ßP (0.1ug/ml) with and without hcG (10 IU/ml) stimulation for 72hrs. S=sulphate, G= glucuronide.

	Control			17,20ßP treated			hcG +17,20ßP treated		
Metabolites	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)	F (ng/ml)	S (ng/ml)	G (ng/ml)
ALDO	2.53±0.35	–	–	–	–	–	–	–	–
F	0.55±0.20	–	–	–	–	–	–	–	–
B	1.18 ±0.23	–	–	1.78±0.07	–	–	–	–	–
DOC	1.01±0.24	–	–	–	–	–	–	–	–
19OHA	1.13±0.18	–	–	–	–	–	–	–	–
11ßOHA	0.73±0.28	–	–	0.25±0.04	–	–	–	–	–
AD	1.30±0.32	–	–	2.48±0.37	–	–	0.46±0.13	–	–
T	1.23±0.26	–	–	0.80±0.17	–	–	–	–	–
17-OHP	–	–	–	1.06±0.16	–	–	–	–	–
17,20ßP	–	–	–	31.7±1.17	27.21±1.39	8.74±1.08	17.07±0.96	38±1.12	10.53±1.07
P4	1.70±0.26	–	–	+	–	–	–	–	–
E	0.16±0.04	–	–	–	–	–	–	–	–
E1	2.30±0.43	–	–	–	–	–	–	–	–
E2	1.35±0.29	–	–	–	–	–	–	–	–
UNK19.2	–	–	–	+	–	–	–	–	–
UNK23.3	–	–	–	+	–	–	–	–	–
UNK31.0	–	–	–	+	–	–	–	–	–
UNK41.0	–	–	–	+	–	–	–	+	–
UNK41.9	–	–	–	+	–	–	–	+	+
UNK44.0	–	–	–	+	–	–	+	–	–
UNK46.5	–	–	–	++	–	–	++	–	–

DISCUSSION

The present work demonstrates that the spawning season of *C. watsoni* and *B. vagra*, lasts much longer (May to August) than was previously observed by Shaikh and Jalali (1991, *C. watsoni*) and Haider (1985, *B. vagra*). The gradual decline in the GSI of the two species reported in this study is not only consistent with asynchronous development of the oocytes in the two species but also with the fact that that the fry of these species are encountered in the streams until August. Furthermore, Zuberi (1990) has also shown that *B. vagra* breeds in the local streams during the months of May to August. The histological observations of the present work also reveal that the ovaries in both species are quiescent in months immediately following spawning (September to November). Recrudescence of the ovaries begins in December and batches of oocytes pass asynchronously through primary growth and secondary growth phases in the succeeding months during which time thecal, granulosa and vitelline envelopes differentiate and vitellogenesis occurs. By April, yolk sequestration is complete in the largest oocytes and maturational progress (germinal vesicle migration and its breakdown) becomes evident marking reinitiation of the meiotic process in preparation for spawning in May onwards. During the spawning season, batches of secondary and tertiary stage follicles that would ultimately undergo maturation sequentially also exist in the ovaries of both species. The *in vivo* steroid hormonal profile obtained in the present investigation is also consistent with the observed GSI and the morphological details for the two species.

Vitellogenic growth of oocytes involving hepatic yolk synthesis and its sequestration as well as maturational events comprising germinal vesicle (GV) migration and its ultimate breakdown (GVBD) are well known to be governed by hormones. The vitellogenic growth is regulated largely by ovarian estrogens and the maturational events mainly by progestogens (Ng and Idler, 1983; Goetz, 1983). The ovarian estrogen that is classically known to regulate synthesis of yolk precursors in the liver and finally their sequestration in the oocytes is estradiol 17β (E_2). As is further elaborated in the following discussion, androgens, particularly testosterone (T), and even interrenal or ovarian corticosteroids are also involved in ovarian developmental events but their role varies in a

species-specific manner. The ovarian progestogen best known as maturation inducing hormone (MIH or MIS) is 17, 20βP with 17-OHP, P_4, T and the 11-oxygenated corticosteroids as equally or nearly effective steroids, at least *in vitro*, in several species (Jalabert, 1976; Goetz, 1983; Ng and Idler, 1983; Greeley *et al.*, 1986; Scott and Canario, 1987; Nagahama, 1987b, c; Canario and Scott, 1987a, b, c, 1988, 1990a; Trant and Thomas, 1988, 1989 a, b; Haider and Inbcraj, 1989; Matsuyama *et al.*, 1990; Haider and Rao, 1992; Petrino *et al.*, 1993; Haider and Balamugran, 1995). It was thus imperative to examine the annual ovarian steroid profile, steroid metabolism *in vitro* and steroid metabolites of cultured oocytes in order to achieve an understanding of the endocrine regulation of reproduction in the two cyprinid species. A variety of ovarian steroids were identified during the annual reproductive cycle of *C. watsoni* and *B. vagra* by solid phase extraction using Sep-Pak C18 and HPLC. Unfortunately, identification of several chromatographic peaks was hampered by non-availability of the desired range of standards.

Solid phase extraction of ovarian steroids was adopted presently as the method of choice in view of the advantages it offers over the rather less efficient liquid-liquid partitioning method (McDonald and Bouvier, 1995). Venkatesh *et al.* (1989) have compared the perfomance of Sep-Pak C18 with other available variations and have found it to be the most productive approach for separating a wide range of steroids with high level of specificity, sensitivity and a recovery rate as high as 90-99%. With this method several estrogens, androgens and progestogens as well as their conjugates can be separated simultaneously from ovarian homogenates (Venkatesh *et al*, 1989; 1992a; Scott and Canario, 1992; Kime *et al.*, 1994; McDonald and Bouvier, 1995), resolved and identified by reverse phase HPLC employing gradient elution. Of the various available methods, HPLC has also been the method of choice for rapid and high degree of resolution of steroids having a wide range of polarity and which may occur *in vivo* or *in vitro* (Shackleton, 1986; McDonald and Bouvier, 1995). Several steroids can be measured simultaneously in a single aliquot and in a short period of time. Caution is, however, warranted in regard to the identity of the steroids resolved in view of the likelihood that other non-steroidal compounds sometimes may have similar elution times (see also,

Khan, 1996). This problem is often overcome by coeluting samples with radiolabeled standards both for identification and for quantification. While local resource constraints prevented such recourse in the present investigation, Venkatesh *et al.* (1989) have demonstrated the high degree of reliability of the method in terms of recoverability and identity of the steroids and their metabolites involving use of both labeled and unlabeled standards.

Steroid biosynthesis and seasonal steroid profile have been examined in the past in diverse marine and freshwater species of teleosts belonging to such major groups as Salmoniformes, Cypriniformes, Cyprinodontiformes, Siluriformes, Perciformes, Atheriniformes and Pleuronectiformes (Fostier *et al.*, 1983; Goetz, 1983; Scott and Canario, 1987; Kime, 1993; Nagahama, 1994a, b; Nagahama *et al.*, 1994; Borg, 1994). The presently available information reveals considerable variations in steroidal profiles, timing of steroid biosynthesis and levels of individual steroids among the various species examined. Some of these differences are clearly associated with the pattern of ovarian development unique to individual species--asynchronous, synchronous or group synchronous. The seasonal profile of the ovarian steroids obtained in the present study on *C. watsoni* and *B. vagra* closely matches the annual GSI and is commensurate with the asynchronous development of the oocytes. The results of *in vitro* responses of ovarian follicles to the various exogenous estrogens, androgens and progestogens given alone or in combination with hCG as well as the metabolites recovered from the incubates provide useful information in respect to both regulation of ovarian development and presence of the requisite enzyme systems in the follicles of the two species.

Estradiol 17β (E_2) is universally well known as the physiologically most potent estrogen in fish as is also the case in other vertebrates. On the other hand estrone (E_1) has been detected in the ovaries of very few species of fish and, along with estriol, has been the least understood in terms of its biological significance. In both *C. watsoni* and *B. vagra,* all of these three estrogens were identifiable during major part of the reproductive cycle. Barring minor differences in their levels in individual months, the general trend in both species was their rising levels during the recrudescent period (vitellogenic progress)

with peaks coinciding with the vitellogenic phase in March and April followed by gradual decline in the succeeding spawning months. In the spent ovaries, these were more or less undetectable. Interestingly also, E_1 turned out to be a particularly conspicuous estrogen and matched E_2 in both species in terms of its persistence and levels during the recrudescent and spawning periods. A profile of E_2 very similar to that of these two species characterizes the spotted sea trout, *Cynoscion nebulosus* (Thomas *et al.*, 1988), which is a multiple spawner with a long spawning season (April to September). In this species, E_2 levels drop by September to only 50% of the April value and T follows a similar pattern. In another serial spawner, the Kanehira bitterling (*Acheilognathus rhombea*: Shimizu *et al.*, 1985), the level of E_2 increases during vitellogenesis and remains high during the maturation phase when vitellogenic oocytes are also being recruited during the repeated 5-day annual cycle. In synchronous spawners such as the salmonids (*Salmo gairdneri*: Scott and Sumpter, 1983; *Oncorhynchus nerka*: Truscott *et al.*, 1986; other salmonids: Simpson and Wright, 1977; *S. trutta*: Kime and Manning, 1982), E_2 drops precipitously at the end of vitellogenesis. In contarst to E_2, the physiological significance of E and E_1 vis-a-vis E_2 in mediating vitellogenic growth of oocytes is difficult to assess and is poorly understood currently. Estrone (E_1) has been reported in a few species only. Eleftheriou *et al.* (1966) have shown that the levels of this steroid in *Ictalurus punctatus* rise 1-3 weeks prior to spawning. Rising levels of E_1 have also been shown to precede spawning in the Atlantic salmon, *Salmo salar* (Cedard *et al.*, 1961) and rainbow trout, *S. gairdneri* (Bohemen and Lambert, 1981). In the trout, both E_1 and E_2 correspond with vitellogenesis but whether E_1 too is physiologically important in this species has remained unclear. Elevated levels of E in the Atlantic salmon occur during spawning (Cedard *et al.*, 1961). In the zebra fish, *Brachydanio rerio* (Van Ree *et al.*, 1977), it is estrone and not estriol or estradiol which stimulate maturation of oocytes. Urist and Schjeide (1961) have claimed that E_1 is involved in induction of vitellogenesis in *Paralabrox clathratus*. In contrast, whereas E_1 has been detected by Pankhurst and Conroy (1988) in the plasma of orange roughy, *Hoplostethus atlanticus*, it does not show any correspondence with seasonal ovarian cycle. In female *Fundulus grandis*, rather low levels of E_1 have been observed without showing any correlation with the annual reproductive cycle (Greeley *et al.*, 1988). Terkahin-Shimony and Yaron (1978) and

Bohemen and Lambert (*S. gairdneri*, 1981) have argued that in teleosts E_1 and E_2 have a synergistic role in promoting vitellogenic growth of oocytes. While the concentration of both E and E_1 increased in the ovary of *C. watsoni* and *B. vagra* during vitellogenesis and declined in the spawning season, experimental evidence would be of considerable value in determining their precise physiological role or possible contribution in vitellogenin sequestration in these species.

Concomitantly with estrogens, the androgens too showed peak concentration in early vitellogenic phase of the follicles (February/March) in both species; the level of AD at this time being markedly higher than that of T in *C. watsoni*. Classically T serves as the substrate for E_2 synthesis and AD for E_1 synthesis in the ovary of vertebrates in general (Gore-Langton and Armstrong, 1994). In both species, the two steroids declined gradually reaching low levels in June (middle of spawning season) that were still appreciably high than in the spent fish. There is no previous report of 19-OHA in any species of fish, and seasonal ovarian pattern of 11ß-OHA has also been rarely studied Both of these steroids also peaked in the initial vitellogenic period in *C. watsoni* and *B. vagra*. 19-OHA declined transiently before rising again at the end of spawning in August, whereas 11β-OHA waxed and waned with peaks in March, June and August. In *B. vagra*, both of these androgens achieved maximum levels in March and decreased to low levels in May (19-OHA) and June (11β-OHA). Interestingly, the levels of these androgens in *B. vagra* were noticeably higher than those of T and AD. Whether these androgens merely represent downstream products of low biological activity or have significance in maintaining viability of oocytes and participate in their maturational progress, particularly in *B. vagra*, needs to be tested experimentally. As stated earlier, the seasonal profile of plasma E_2 and T in the teleost species varies depending on the pattern of their seasonal reproductive cycle, synchronous or asynchronous ovarian development. In several species, both of these steroids generally show increasing levels that are principally coincident with vitellogenic progress but continue to rise even during the spawning season in some species regardless of the temporal pattern of ovarian development (*S. gairdneri*: Scott *et al.*, 1983; *Catostomus commersoni*: Scott *et al.*, 1984; *Acheilognathus rhombea*: Shimizu *et al.*, 1985; *Cyprinus carpio*: Yaron and Levavi-

zermonsky, 1986; *Oncorhynchus nerka*: Truscott *et al.*, 1986; *Fundulus heteroclitus*: Bradford and Taylor, 1987; *Hoplostethus atlanticus*: Pankhurst and Conroy, 1988; *Cynoscion nebulosus*: Thomas *et al.*, 1988; *Limanda yokohamae*: Hirose *et al.*, 1988; *Gobio gobio*: Rinchard *et al.*, 1993; *Stizostedion vitreum*: Malison *et al.*, 1994; *Anguilla japonica*: Ijiri *et al.*, 1995; *Dicentrarchus labrax*: Asturiano *et al.*, 2000). In fact, in *Stizstedion vitreum*, T shows a bimodal rise. The first peak occurs prior to spawning and the second at spawning. In this species, 11-ketotestosterone has also been detected without evidence of seasonal fluctuations. King *et al.* (1995) have found that maximum levels of plasma E_2 and T in white perch (*Morone americana*) and white bass (*Morone chrysops*) continue to prevail during GV migration and GVBD before declining during the hydration and ovulation period. Both of these species, are multiple batch spawners as are the two species studied presently. Such species differences as enumerated above in the timing of E_2 and T or their relative roles in vitellogenesis and maturation even within the salmonid group are also on record. In coho salmon, *Oncorhynchus kisutch*, E_2 production is highest in fully grown follicles while T is highest during GV migration (Van Der Kraak and Donaldson, 1986). A similar sequence has been demonstrated in amago salmon ovarian follicles (Kagawa *et al.*, 1983a; Young *et al.*, 1983b, c). In contrast, plasma E_2 in *Sardinops melanostictus* has been shown to reach peak levels by the end of spawning, whereas T remains undetectable throughout the year (Matsuyama *et al.*, 1991).

The androgen known to be most widely distributed in the ovary of fish is T, and often its level is higher than encountered in the testis. Compared to T, considerably less attention has been directed at identification of or analysis of seasonal variations in AD, 11β-OHA, 19-OHA, 11β-OHT and 11-KT in ovaries of fish. 11-KT is the major androgen in the male sex (Idler *et al.*, 1960; Katz and Eckstein, 1974; Kime, 1979a; *P. platessa*: Campbell *et al.*, 1976; Simpson and Wright, 1977) and 11β-OHT in gonochristic and ambisexual teleosts (Idler *et al.*, 1971, 1976). Where examined (*Salmo gairdneri*: Scott and Baynes, 1982; *Catostomus commersoni*: Scott *et al.*, 1984), AD follows the same pattern as T with peak levels coinciding with vitellogenic progress. Whether some of the unidentified peaks in the chromatograms represented 11-KT and

11β-OHT in the follicles of *C. watsoni* and *B. vagra* can only be settled after further investigations. AD has remained undetectable in the ovary of *Tilapia aurea* (Eckstein and Katz, 1971), affirming the variability of species in terms of steroidogenesis. In *C. watsoni*, significant increase in AD preceded the increase in T (February and March respectively). Although the levels of both dropped and differed slightly from the pattern noted in *B. vagra* in April, these were still appreciably high until June in contrast to the spent fish. In *B. vagra*, both T and AD increased in parallel in February and March coincident with the initial vitellogenic period and remained comparable even in May (spawning fish) when maturing oocytes coexist with vitellogenic oocytes. However, interestingly, 19-OHA and particularly 11β-OHA were the most conspicuous steroids in March to May showing several fold increase over their January values (early recrudescent phase). On the other hand, while the levels of T and AD were declining in the spawning season, 11β-OHA showed a second peak in mid spawning (June) and at the end of spawning (August). 19-OHA also increased sharply again in August in *C. watsoni*. Unfortunately, very scanty information exists in the literature on the occurrence, seasonality and biological role of these androgens (Kime, 1979a; Fostier *et al.*, 1983; kime, 1993) and hence it is difficult to analyze their precise involvement in the ovarian regulatory events. 11β-OHA has been identified in the ovary of *Anguilla anguilla* (Kime, 1979a) and deemed to be the main 11β-hydroxylated steroid in the ovary of gray mullet (*Mugil cephalus:* Azoury and Eckstein, 1980), where it does not change with advancement of ovarian development. In general, a fall in ovarian androgen levels at the conclusion of vitellogenesis and by onset of maturation is a typical feature of synchronous batch spawners (*S. gairdneri*: Scott *et al.*, 1983; *Stizostedion vitreum*: Pankhurst *et al.*, 1986; *Oncorhynchus gorbuscha*: Dye *et al.*, 1986; *S. gairdneri*: Liley *et al.*, 1986). This has been thought to result from diversion of their precursors towards synthesis of 17, 20βP or synthesis of some downstream androgens (11-oxygenated androgens). However, it is not obligatory that androgen levels fall precipitously prior to oocyte maturation. In fact, in the case of coho salmon (*Oncorhynchus kisutsch*: Fitzpatrick *et al.*, 1986), the levels of T and 11-KT remain high throughout maturation and ovulation. That there are considerable species variations among female teleosts in regard to the behaviour of T in ovarian development is evident from several studies. A

preovulatory peak in T has been reported in the synchronous spawners such as salmonids (Fostier and Jalabert, 1986). In sockeye salmon (synchronous spawner), the levels of T decrease significantly only after spawning (Truscott *et al.*, 1986). In the catfish, *Clarias gariepinus*, T peaks at postvitellogenesis and prespawning stage *in vitro* (Schoonen *et al.*, 1987). It has been shown that T also has a role in preparing the oocytes for final maturation and GVBD *in vitro* (*Brachydanio rerio*: Van Ree *et al.*, 1977; *Oncorhynchus rhodurus*: Nagahama *et al.*, 1980; Young *et al.*, 1982). In landlocked salmon, T has been shown to stimulate GV migration (So *et al.*, 1985). In an earlier study on the goldfish, *C. auratus*, T has been thought to promote survival of postvitellogenic follicles and to induce maturation of oocytes *in vitro* (Remacle, 1976; also Stacey *et al.* 1983). Such a role has also been suggested by several workers in other species (*O. latipes*: Iwamatsu, 1978; *H. fossilis*: Lamba *et al.*, 1983; *F. heteroclitus*: Greeley *et al.*, 1986; *Catostomus commersoni*: Scott *et al.*, 1984). The highest levels of T in the ovarian follicles have been recorded in the spawning period when there is a precipitous drop in the E_2 levels in the plaice, *P. platessa* (Wingfield and Grimm, 1977) and the Indian catfish, *H. fossilis* (Sundraraj *et al.*, 1982).

A variety of progestogens have been described in the past in the plasma of female fish or have been detected *in vitro* following exposure of follicles to various steroid substrates indicating intraovarian availability of enzyme systems necessary for their synthesis. In the present study, non availability of a full range of standards made possible identification of only three progestogens, P_4, 17-OHP and 17, 20βP. All of these cycled during the year in the two species, paralleled the increase in estrogens and androgens and reached peak levels in April and May (*C. watsoni*) or March and April (*B. vagra*). While P_4 remained at comparatively low levels throughout the year (particularly in *B. vagra*), the concentration of 17-OHP and 17, 20βP was several fold higher than that of the estrogens and the androgens in the two species. Equally interesting is the observation of persistently and noticeably elevated levels of these progestogens in the spawning months (May, June and July) when successive batches of oocytes are ready for maturation and ovulation coincident with gradually declining levels of the estrogens, T and AD. Furthermore, almost 3-fold higher concentration of 17,20βP in *B. vagra* in March and

April compared to its concentration in *C. watsoni* reflects a notable species difference. The relatively much lower levels of P_4 than those of 17-OHP and 17, 20βP in both species for greater part of the year suggest that either its synthesis is limited or it is rapidly converted into its downstream metabolites of 17-OHP and 17, 20βP via 17α-hydroxylase and 20β-HSD, respectively. In the Kanehira bitterling too, P_4 has not been found to show significant change during the reproductive cycle, while both 17-OHP and 17,20βP rise during the maturation phase; P_4 acting mainly as a substrate for 17,20βP (Shimizu *et al.*, 1985). P_4 is the principal substrate for the various gonadal steroids and has hardly any regulatory significance during the cyclic ovarian development (Kime, 1993). The view that 17,20βP is the most potent MIS in fish emerged principally from studies on salmonids where both *in vivo* and *in vitro* presence of this progestin at high levels in maturing oocytes has been recorded in a number of species (reviews: Kime, 1993; Scott and Canario, 1987). Its high levels in the periovulatory season provide sufficient grounds to suggest that in *C. watsoni* and *B. vagra* too it is the principal MIH *in vivo*, with P_4 and 17-OHP merely serving as its precursors.

Several corticosteroids and deoxycorticosteroids (progestogens with OH group at positions 21/11 and at 21 only, respectively), presumably of interrenal origin, have been described in the past in the plasma of various species of fish (*Oncorhynchus tshawytcha*: Robertson *et al.*, 1961; *Oncorhynchus nerka*: Schmidt and Idler, 1962; *C. auratus*: Honma and Tamura, 1965; *Gadus morhua*: Woodhead and Woodhead, 1965; *O. nerka*: McBride and Overbeeke, 1969; *S. gairdneri, C. auratus, E. lucius*: Jalabert, 1976; *P. platessa*: Wingfield and Grimm, 1977; Colombo *et al.*, 1978a,b; *C. auratus:* Peter *et al.*, 1978; Cook *et al.*, 1980; *S. trutta*: Pickering and Christie, 1981; *O. rhodurus*: Young *et al.*, 1982; *H. fossilis*: Sundraraj *et al.*, 1982; Goswami and Sundraraj, 1974; *O. kisutch*: Morrison *et al.*, 1985; *O. mykiss*: Contreras-Sanchez *et al.*, 1995; *C. harengus pallasi*: Carolsfeld *et al.*, 1996) with their concentration or identity varying from species to species as well as season. In the present investigation only three of these, DOC (11-deoxycorticosterone, 21-hydroxypregn-4-ene-3, 20-dione), B and F, were identifiable in the two species throughout the year. Their presence in the ovarian tissue reveals presence of the requisite enzyme systems and their active production within the ovary; the

synthesis of F being noticeably low throughout the year in both *C. watsoni* and *B. vagra*. DOC and B were dominant in the two species with notable species differences. In *C. watsoni*, DOC appeared to be associated with the recrudescent and vitellogenic phases of the follicles showing its high levels in this extended period. In *B. vagra*, it was dominant in the periovulatory period (February to May), fell sharply in June and again peaked in July (late spawning time) suggesting association with both vitellogenesis and maturation of the oocytes. Interestingly, the level of DOC was nearly 2-fold greater than that of B in this species. Both B and F started increasing in the recrudescent phase, peaked in March (mid-vitellogenesis) and fell precipitously in June (mid spawning). Significantly higher levels of F prevailed in *B. vagra* in March/April than in *C. watsoni*. The role of corticosteroids and deoxycorticosteroids in reproductive physiology of fishes has been of interest especially in view of the initial demonstration *in vitro* by Goswami and Sundraraj (1971a, b, 1974) that both 11-deoxycorticosterone (DOC) and 11-deoxycortisol (17, 21-dihydroxy-4-pregnen-3, 20-dione: 11 DOC) prevail throughout the year in the Indian catfish, *Heteropnuestes fossilis* and represent the most potent MIS *in vivo* and *in vitro*; a view that has been negated by further studies on this and other catfish (*H. fossilis*: Ungar *et al.*, 1977; Sundraraj *et al.*, 1985; *M. vittatus*: Upadhyaya and Haider, 1986). In female sockeye salmon, *Oncorhynchus nerka*, high levels of plasma 11-deoxycortisol prevail throughout the year with peak levels in postspawned fish, whereas DOC levels remain consistently low throughout (Truscott *et al.*, 1986). 11-deoxycorticosteroids are also known to be produced in the ovaries of several other teleosts (*Pseudopleuronectes americanus*: Campbell *et al.*, 1976; *Dicentrarchus labrax*: Colombo *et al.*, 1978b; *Clupea harengus pallasi*: Carolsfeld *et al.*, 1996) and are capable of inducing maturation of the oocytes *in vitro*. In the herring, plasma DOC profile is consistent with maturation but its MIS effectiveness has been considered to be much less than that of 17,20βP (Scott and Canario, 1987; Asahina *et al.*, 1992). In the African catfish, *Clarias lazera*, DOC is not produced by the ovarian follicles *in vitro* and hence has not been regarded as MIS (Lambert and Van den Hurk, 1982). DOC and 11-DOC have been reported to be absent in the carp (*C. carpio*) during postvitellogenetic phase but have been shown to correlate with ovulatory and postovulatory phases in *Dicentrarchus labrax* (Colombo *et al.*, 1978b). DOC is also known to enhance action of GTH and 17, 20βP in amago salmon, *O.*

rhodurus (Young *et al.*, 1983a) but F is ineffective in this respect. In goldfish, *Carassius auratus*, 11-deoxycortisol (11-DOC) is second to 17, 20βP in causing GVBD *in vitro* (Jalabert, 1976). Increasing concentrations of plasma cortisol (Cook *et al.*, 1980) and cortisone have been shown during oocyte maturation, near ovulation and spawning in several teleosts including sockeye salmon (*O. nerka*: Schmidt and Idler, 1962; *S. trutta*: Pickering and Christie, 1981). In the flounder, *Pseudopleuronectes americanus:* cortisol is the major C 21 steroid, whereas cortisone, corticosterone (B), 11-deoxycorticosterone (DOC) and 11-dehydrocorticosterone are minor steroids (Campbell *et al.*, 1976; Colombo *et al.*, 1978a). Bradford and Taylor (1987) have found elevation in serum F in association with ovulation and have observed a surge at spawning in *F. heteroclitus*. In the ovarian fluid of this species, only low levels of F could be detected presumably due to its poor access from the plasma into the ovaries. In the medaka, *Oryzias latipes*, Hirose (1972) has shown F to be effective in ovulation and has suggested that corticosteroidogenesis occurs in the follicular tissue of this species. In *H. fossilis*, the levels of F remain high during recrudescence of the ovary (Sundraraj *et al.*, 1982) where it enhances estrogen-induced vitellogenin synthesis. According to Lamba *et al.* (1982), T, E_2 and F are the principal plasma steroids in *H. fossilis*. F elevates in the preparatory and prespawning seasons and matches the rise in E_2 and vitellogenin in this species. High levels of cortisol have also been recorded in female goldfish (Peter *et al.*, 1978; Honma and Tamura, 1965; Woodhead and Woodhead, 1965; Fuller *et al.*, 1976). Cortisol provides energy by increasing peripheral proteolysis, fat release and utilization of hepatic gluconeogenesis amd hence plays a role in stress. Jalabert (1975, 1976) has argued that F has a sensitizing effect on MIS, and according to Wallace and Selman (1978,1980) F helps in recruitment of follicles from late vitellogenesis to maturation. In their view, high levels of plasma F during spawning reflect a stress response to modify metabolism but they could not detect elevation of glucose in the plasma at any time during the year. Whether the elevation of F in *C. watsoni* in August (when spawning is over) is a stress-associated response and/or has direct association with vitellogenesis and maturation is difficult to judge without additional experimental work. According to Haddy and Pankhurst (1999), plasma F remains unaltered throughout the year in black bream, *Acanthopagrus butcheri* but stress causes drastic elevation in plasma F. Ruane *et al.* (2001) have shown that stress results in

increase in the levels of corticosteroids in the plasma of common carp. Confinement of fish in nets causes rapid increase in circulating F and its return to basal level on removal of the stress. Similar observations have been made by Pottinger (1998) and Tanck *et al.* (2000) on the common carp. There is little doubt in the light of the available information on corticosteroids under discussion that there is great species variation and that much in-depth work is needed to elucidate the relative roles of these steroids in reproduction.

In addition to estrogens, androgens, progestogens, corticosteroids and deoxycorticosteroids, a peak coeluted with aldosterone standard throughout the reproductive cycle of *C. watsoni* and *B. vagra*. According to Colombo *et al.* (1978a), aldosterone (18, 11-hemiacetal of 11β, 21-dihydroxy-3, 20-dioxo-4-pregnen-18-al) does not occur in teleosts. On the other hand, Coimbra *et al.* (1996) and Khan (1996) have reported its presence in some teleosts. The steroid, presumed here to be ALDO, increased significantly in the spawning period over and above its levels in the postspawning season and declined in the quiescent ovaries in both species. Its tentative identity as ALDO as well as its physiological significance in reproduction requires further work. Theoretically, the formation of ALDO is possible from P_4 via corticosterone by the enzyme P450 11-hydroxylase ($P450_{c11}$), also called aldosterone synthetase ($P450_{aldo}$).

The steroid profile of the two species collectively reveals that while the estrogens, androgens and progestogens, in general, reach their peak levels in March and April, the progestogens (particularly 17-OHP and 17, 20βP) persist at high levels for a longer duration during the spawning season in concert with gradually declining levels of the estrogens and the androgens. In species where the follicles develop synchronously followed immediately by ovulation and spawning, the estrogens (e.g. estradiol 17β) and the progestogens (e.g. 17,20βP) have more clearly defined temporal sequencing, the former peaking around vitellogenesis and the latter with a delay when the oocytes are entering the maturation phase. In species where batches of oocytes are simultaneously in vitellogenic and maturation phases as is the case in asynchronous development of follicles (serial/multiple spawners) in the two species, such a clear separation of peaks would be rather uncharacteristic. High levels of estrogens in the initial period of

vitellogenesis (February to April) and persistently high levels of 17, 20βP in the months of April to July when batches of maturing oocytes coexist with vitellogenic oocytes, are features that are consistent with a pattern characteristic of other asynchronous spawners as well. Although T is a substrate for E_2 and an intraovarian modulator of GTH action on aromatase prior to final maturation (Hirose *et al.*, 1988), it has also been considered to promote maturation of the oocytes through GTH-II synthesis in a number of species of fish, whether asynchronous or synchronous spawners (*Bracydanio rerio*: Van Ree *et al.*, 1977; Crim and Idler, 1978; several teleosts: Nagahama *et al.*, 1982; *C. auratus*: Kagawa *et al.*, 1983a; Nagahama *et al.*, 1993; So *et al.*, 1985; *C. carpio*: Yaron and Levavi-Zermonsky, 1986; Kobayashi *et al.*, 1988a; *Cynoscion nebulosus*: Thomas *et al.*, 1988; *S. melanostictus*: Matsuyama *et al.*, 1991). Its high levels well into the maturation phase have also been demonstrated in *Anguilla japonica* (Ijiri *et al.*, 1995), sturgeon hybrid (Mojazi Amiri *et al.*, 1996) and sea bass, *Dicentrarchus labrax* (Asturiano *et al.*, 2000). The ability of T to promote GVBD *in vitro* is evident in *C. watsoni* and *B. vagra* as well (see below) though at relatively high dosage level. P_4 is a substrate for 17-OHP and its relatively low levels in both species may be due to its rapid metabolization into this steroid via 17-hydroxylase or that its synthesis is somewhat limited in the two species. 17-OHP is not only a substrate for synthesis of 17, 20βP or can be converted into T through C21 to C19 desmolase (Scott *et al.*, 1984) but itself is nearly as potent MIS *in vitro* as 17, 20βP in many teleosts (See Goetz, 1983; Scott and Canario, 1987; Canario and Scott, 1988; kime, 1993; Scott *et al.*, 1984, 1998), as has also been demonstrated presently for *C. watsoni* and *B. vagra*. The declining levels of this steroid concomitant with persistently high levels of 17, 20βP during the spawning season, however, suggest its gradual conversion into the latter in these species but its role as MIS cannot be entirely ignored at the present time.

In vitro study of gonadal responses to exogenous hormones has yielded information of immense value in understanding reproductive regulatory mechanisms and metabolism of steroids and in providing direct evidence of existence of the repertoire of enzymes necessary for their biosynthesis. Earlier *in vitro* investigations by Ghaffar (1988), Zuberi (1990) on *B. vagra* have provided preliminary evidence of the

responsiveness of ovarian follicles of this species to selected steroids, hCG and carp pituitary homogenates. The present investigation extends this work and shows that exogenous hCG, E_1, E_2, AD, T, DOC, 17-OHP, P_4 and 17, 20ßP are capable of influencing the behaviour of the germinal vesicle *in vitro* but only the androgens and the progestogens are the most effective steroids causing its breakdown (GVBD). The response of the follicles is, however, both dose-dependent and time dependent. Thus, at the highest dosage of 1.0 ug/ml, all of these steroids elicited maturational response that was maximal when the follicles were exposed for 72 hr. The response diminished when both the time of exposure to these steroids as well as dosage was reduced but under this circumstance (0.01 ug/ml and 24 hr) only 17, 20βP brought about significant GVBD in both species. 17, 20βP, first identified in the plasma of female *O. nerka* by Idler *et al.* (1960), is now considered to be the most potent MIS in a large number of teleosts (*S. gairdneri:* Fostier *et al.*, 1973, 1981; Campbell *et al.*, 1980; Scott *et al.*, 1982; Fostier and Jalabert, 1982; *O. rhodurus*: Young *et al.*, 1983a, b; Nagahama et *al.*, 1980, 1983; Nagahama and Adachi, 1985; Nagahama, 1987b, c; Van Der Kraak *et al.*,1989; *C. auratus:* Jalabert, 1976; *Perca flavescens:* Goetz and Theofan, 1979; *O. latipes:* Iwamatsu, 1980; *Oncorhynchus kisutch:* Sower and Schreck, 1982; *O. rhodurus:* Young *et al.*, 1982; Nagahama *et al.*, 1993; the ayu, *Plecoglossus altivelis:* Nagahama *et al.*, 1983; catfish, *Mystus vittatus*: Upadhyaya and Haider, 1986; *C. batrachus*: Haider and Rao, 1992; mummichog, *Fundulus heteroclitus*: Greeley *et al.*, 1986; Petrino *et al.*, 1993; Atlantic croaker: Trant and Thomas, 1988; cyprinids, *Cyprinus carpio, Labeo rohita, Cirrhinus mrigala* and *Catla catla*: Epler, 1981a, b, Inbaraj and Haider, 1988; Haider and Inbaraj, 1989; the Japanese whiting, *Sillago japonica:* Matsuyama *et al.*, 1990; *Limanda limanda* and *Pleuronectes platessa*: Canario and Scott, 1990a,b; *Morone saxatilis*: king *et al.*, 1993; *C. harengus pallasi*: Carolsfeld *et al.*, 1996). Gonadal steroidogenesis in fish progresses under the influence of two pituitary gondotropins, GTH-I and GTH-II. The former governs vitellogenesis and the latter controls maturation (Goetz, 1983; Nagahama, 1987a,b; Peter, 1981; Swanson, 1991). Estradiol 17β (E_2), produced by aromatization of testosterone (T) under the influence of GTH-I, enters the blood and initiates hepatic synthesis and release of vitellogenin which is then sequestered in the developing oocytes (Wallace and Selman, 1981; Wallace, 1985). In salmonids, both

special thecal cells and granulosa cells are involved in this synthesis (2-cell type model: Kagawa *et al.*, 1985; Nagahama, 1983, 1987a, b; Adachi *et al.*, 1990). In non-salmonid fish, only the granulosa cells are involved in synthesis of T and its aromatization to E_2 (Onitake and Iwamatsu, 1986; Petrino *et al.*, 1989; Nagahama *et al.*, 1994). The results of *in vitro* studies attempting to assess the effectiveness of gonadotropins on follicular maturation have yielded rather equivocal information, sometimes even on the same species. According to some earlier work (Jalabert *et al.*, 1972; Jalabert and Breton, 1973; Sakun and Lemanova, 1976), mammalian gonadotropins are ineffective in inducing maturation of oocytes. Other workers have shown that hCG, LH and mammalian pituitary extracts as well as progesterone and testosterone have strong MIS effect *in vitro* on the ovarian follicles of several species (*Plecoglossus altivelis* and *Heteropnuestes fossilis*: Goswami and Sundararaj, 1971a, b; *Oryzias latipes*: Hirose, 1976; Iwamatsu and Katoh, 1978; *Brachydanio rerio*: Van Ree *et al.*, 1977; salmonids: Jalabert *et al.*, 1974; Nagahama *et al.*, 1980; Goetz, 1976, 1983; Scott and Canario, 1987; *F. heteroclitus*: Greeley *et al.*, 1986; Wallace *et al.*, 1987). In both *C. watsoni* and *B. vagra,* the effect of hCG and various steroids was quite consistent and demonstrates that not only hCG alone promotes maturation but also a 6-hr treatment with hCG combined with different steroids brings about time-dependent improvement in GVBD response. Such a response was especially notable in the case of androgens and progestogens. Epler (1981a,b) and Epler *et al.* (1981/82) have also demonstrated that in the cyprinid, *Cyprinus carpio,* hCG synergises the effect of various steroids on the follicles and promotes the GVBD response significantly. GTH synergies the effect of T on maturation of oocytes in rainbow trout (Jalabert, 1976) and amago salmon (Young *et al.*, 1982).

Some of the past studies aimed at analysis of relative roles of individual steroids revealed that E_2 is capable of inducing oocyte maturation *in vitro* in the Japanese medaka (*O. latipes*: Hirose, 1972,1976), an observation later negated by the work of Iwamatsu (1978) on this species. It seems that conflicting observations on effects of given hormones within a species are liable to stem from the variable culture conditions in different laboratories or on the competence of the oocytes to respond at given times (Kime, 1993). As stated earlier, in the zebrafish, *B. rerio* (Van Ree *et al.*, 1977), it is E_1

that has GVBD stimulatory effect and both E_2 and E are ineffective. More recent investigations have demonstrated that E_2 is incapable of causing maturation of oocytes *in vitro* in most species of fish and the present observations on *C. watsoni* and *B. vagra* also affirm this view. That the estrogens nevertheless have indirect involvement as inhibitors of oocyte maturation and ovulation was proposed in some earlier studies (goldfish: Pandey and Hoar, 1972; rainbow trout: Jalabert, 1975; the loach, *Misgurnus anguillicaudatus*: Ueda and Takahashi, 1976; Indian catfish: Sundararaj *et al*, 1979), implying that while E_2 can regulate maturation, it does not induce it. In contrast to estrogens, T, AD, P_4, 17-OHP, 17, 20βP and also 20βS (20β-dihydroxyprogesterone) have been demonstrated to have strong but species-specific MIS effect *in vitro*. Both the corticosteroids and the deoxycorticosteroids (DOC, 11-DOC) and even F are also known to initiate maturation of follicles *in vitro* in some species (reviews: Goetz.1983; Scott and Canario, 1987; Nagahama, 1994a). Whether the response of the oocytes in different species to at least some of these steroids is direct or ensues from their conversion to more active forms at the follicular level has been a subject of debate for some time (Goetz, 1983; Nagahama, 1987a, b; Scott and Canario, 1987).

Whereas 17, 20βP is now well known to be the most potent MIS in majority of fish, other C-21 steroids have also been found to be equally potent in some species. Among the C-21 steroids, the effectiveness of P_4, DOC and 11-DOC as well as other corticoids and corticosteroids during natural oocyte maturation has been quite controversial. In the Japanese medaka, *Oryzias latipes*, F has been shown to be four times more effective than P_4 in causing maturation of the oocytes (Hirose, 1972), and in the zebra fish (*Brachydanio rerio*) it has been found to be a better MIS than 17, 20βP (Van Ree *et al*., 1977). Goswami and Sundraraj (1971a, b, 1974); Sundraraj and Goswami, (1966) demonstrated that DOC, 11-DOC and 21-deoxycortisol are the strongest MIS in the Indian catfish, *H. fossilis* with P_4, 17-OHP and 17-dihydroxyprogesterone being poor inducers of maturation *in vitro*. While Masui and Clarke (1979) have argued that the observed effect of corticosteroids is unspecific, later works by Ungar *et al*. (1977) and Sundraraj *et al*. (1985) on this species have failed to confirm the earlier observation and have demonstrated that 17, 20βP is physiologically the most potent MIS in *H. fossilis*

(see also African catfish, *Clarias gariepinus*: Lambert and Van den Hurk, 1982). On the other hand, 11-DOC, hydrocortisone and F have been demonstrated to be ineffective as MIS in rainbow trout (Jalabert *et al.*, 1972; Fostier *et al.*, 1973), chum salmon (Osanai *et al.*, 1973) northern pike (Jalabert and Breton, 1973). While 17, 20βP, 20βS and 11-DOC are all effective in causing GVBD in the brown trout (Duffey and Goetz, 1980) and the yellow perch (Goetz and Theofan, 1979), 17,20βP remains the most potent of these.

In the present study too, T, AD, P_4 and 17-OHP induced maturation of intrafollicular oocytes *in vitro* but had a significant effect mainly at high concentrations or when the duration of exposure *in vitro* was increased. 17, 20βP alone brought about the most significant GVBD at the lowest concentration of 0.01 ug/ml and thus it appears to be the physiologically important MIS in *C. watsoni*. In the case of *B. vagra* only a single concentration (1.0 ug/ml) was tried in the present work. Judging from the duration of exposure and the relative GVBD responses of the follicles of this species to the different steroids, 17, 20βP appears to be the MIS that may be physiologically the most important. As discussed by Trant and Thomas (1988), apparently the side chain of the progestin molecule is a key structural component of the MIS that interacts with the active site of the oocyte receptor controlling final oocyte maturation. Certain alterations of the side chain of progesterone increase the potency of the resultant steroid 1000 fold. Trant and Thomas (1988) have shown that steroids with hydroxyl groups at the 17α, 20ß or 21 position are more potent than P_4, particularly at concentrations below 0.01 ug/ml. 11-deoxycorticosterone (DOC, where hydroxylation of P_4 is at the 21 position) is more potent than 17-OHP (hydroxylation of P_4 at the 17α position). The 17, 20ß-dihydroxy configuration of P_4 (17α, 20ß-P4) results in maximal stimulation of GVBD at near physiological concentration.

One of the attempts in the present study was to identify the steroid metabolites released into the incubation medium following exposure of intrafollicular oocytes (central GV stage) of *C.watsoni* to hCG and different steroids alone, and hCG in combination with different progestogens. *In vitro* studies employing gonadotropins and/or exogenous steroid precursors have been of much value in elucidating gonadal steroid metabolism

and the enzyme systems involved therein (Kime, 1979a, b; Yaron and Barton, 1980; Fostier *et al.*, 1981a, b; Suzuki *et al.*, 1981a, b; Yaron *et al.*, 1982; Kagawa, 1982; Kagawa *et al.*, 1982a, b; Nagahama and Kagawa, 1982; Nagahama *et al.*, 1983; Yaron *et al.*, 1983; Petrino *et al.*, 1989; Kime, 1990, 1992, 1993; Kime *et al.*, 1991, 1992, 1993, 1994; Kime and Abdullah, 1994). The present study provides evidence of the presence of enzymes that can metabolize androgens, estrogens and progestogens during the ovarian cycle of *C. watsoni*. The pattern of the products recovered following *in vitro* incubation of the vitellogenic ovarian follicles with exogenous substrates and the results of the *in vivo* work suggest that the enzyme systems comprising at least 17-HSD, P450 arom, P450 lyase, 20β-HSD, 16α-hydroxylase, 11β-hydroxylase, sulphuronyl transferase and glucuronyl transferase are actively involved in steroid metabolism in the ovarian follicles of *C. watsoni*. Fig. 24 Summarizes the key pathways based on the *in vitro* work reported here.

Incubation of the follicles of *C. watsoni* with hCG alone resulted in the increased production of estrogens, testosterone and corticosterone. Although 17, 20ßP and 17-OHP were also detectable in the medium, in contrast to their absence in the control incubate, their levels were extremely low. It is thus evident that hCG stimulates synthesis of testosterone and estrogens as is expected in view of the role of gonadotropins in promoting production of T and its aromatization to E_2 by P450 arom. Kagawa *et al* (1983a, b) and young *et al.* (1983b, c) have shown that purified salmon gonadotropin (SG-100) stimulates both T and E_2 production by vitellogenic follicles *in vitro* in amago salmon. In goldfish too, hCG enhances synthesis of E_2 *in vitro* in the primary and secondary yolk stage follicles (Kagawa *et al.*, 1984). Enhanced production of 17-OHP and 17, 20βP under the influence of hCG has also been observed in other species but the levels of the individual metabolites produced depends on the stage in which the oocytes may be at the time of incubation. In Atlantic salmon, *Salmo salar* (Zhoa and wright, 1985), GTH markedly stimulates the steroid output in general, where 17, 20βP is released in significantly greater amount. Apparently, the 20β-hydroxysteroid dehydrogenase (20β-HSD) pathway of 17-OHP to 17, 20βP as well as the aromatization pathway is stimulated by hCG. The detection of low levels of 17-OHP and 17, 20βP in the incubate of *C.*

watsoni in response to hCG suggests that the enzyme 20β-HSD could not be activated fully in oocytes that were still mostly in central GV stage at the time of incubation with hCG alone and continued to produce only basal levels of these progestins under its influence (see also Suzuki *et al.*, 1981a, b; Zhao and Wright, 1985). In contrast to the low level of 17, 20ßP, 17-OHP was released in slightly greater amount following hCG stimulation. Only marginal GVBD increase occurred at 72 hr (see Table 14b) when the follicles received only hCG.

When the oocytes were exposed separately to the two estrogens (E_1 and E_2), a substantial interconversion was evident in addition to appearance of their glucuronide and/or sulphated conjugates as well as an unknown steroid, indicating activation of 17-hydroxysteroid dehydrogenase (17-HSD), glucuronyl and sulphuronyl transferases. Since only very low quantities of 17-OHP were produced and 17,20βP was absent in the medium, only margional GVBD was evident in either case (see Table 14b).

The products formed following incubation of the oocytes with exogenous testosterone also suggest a predominance of 17-HSD activity as indicated by the high level of AD and significant disappearance of T from the incubation medium. A significant increase in conversion of T to AD has also been demonstrated in incubates of ovarian fragments of *P. latipinna* (Kime and Groves, 1986), testicular tissue of the African lungfish, *Neoceratodus forsteri* (Joss *et al.*, 1996) and Arctic charr embryos (Khan, 1996). Evidently, aromatase was also activated significantly causing conversion of T to estrogens, which were also metabolized by the appropriate transferases into their conjugates. The presence of substantial quantity of E_1 in the medium following incubation of the oocytes with AD confirms activation of the AD to E_1 pathway while a significant proportion of the substrate remained unmetabolized even after 72 hr. AD is the principal substrate for E_1. The presence of some unknown compounds (presumably downstream androgens) warrants further studies to determine their precise identity. Querat *et al.* (1986) and Lambert *et al.* (1991) have observed that the ovarian slices of the European eel (*Anguilla anguilla*) convert AD mainly to 5ά-androstenedione (5ά-

androstane-3, 17-dione) and 3β-hydroxy-5α-androstane-17-one (androsterone) representing the 5α-reduced metabolites. The unknown compounds noted in the incubate of *C. watsoni* may also be 5α-reduced steroids such as 5α-androstane-3, 17 dione, androsterone and epiandrosterone. The fact that a small amount of the precursor was converted to T and E_2 suggests weak 17ß-HSD type-I activity, besides strong 5α-reductase activity. In addition to 17ß-HSD isozyme type-2, other enzymes such as P450 arom may be active in converting T and AD to E_2 and E_1, respectively. This activation of P450arom activity is necessary for promoting steroidal regulation of vitellogenic growth. Rodriguez Maldonado *et al.* (1996) working on the thecal cells of preovulatory follicles of the domestic hen (*Gallus domesticus*) and Khan (1996) on Arctic charr embryo have shown preferential formation of E_1 rather than E_2 following incubations with AD, and predominant formation of AD following incubation with T. Akinola and coworkers (1996) have proposed that various 17ß-HSD isozymes in rat regulate exposure of tissues to androgens and estrogens by converting them into their biologically less potent forms. The formation of sulphate or glucuronide conjugates of the two substrates by sulphuronyl transferase and glucuronyl transferase, respectively, in the present study not only provides protection of the follicles against unusual exposure of the oocytes to the free active steroids but also facilitates their excretion (see also Hobkirk, 1993).

In *Cyprinion watsoni*, gonatotropin stimulation of ovarian follicles before adding progesterone (see Table 18) as a substrate enhanced production of 17-OHP and 17,20ßP over and above the control incubation (where these were below detection limit). As the concentration of 17-OHP also increased with gonadotropin stimulation, it is possible that the enzyme responsible for the conversion of P_4 to 17-OHP (by 17α-hydroxylase) became more active which was then converted in turn to 17, 20ßP by activating 20ß-HSD, the enzyme involved in the conversion of 17-OHP to 17,20ßP (Gore-Langton and Armstrong, 1994). The present results indicate that gonadotropin stimulation enhances the metabolism of P_4 (~96% P_4 metabolized in 72 hrs as compared to ~63% conversion in its absence, Table 23). The incubation of the ovarian follicles with P_4 resulted in slight increase in the production of DOC, 17-OHP, 17,20ßP, T, E_1, E_2, 11ß-OHA, B (corticosterone) and some other steroids (unknown peaks). This has also been

demonstrated in the case of *F. heteroclitus* by Petrino *et al.* (1993) who identified 17-OHP, 20ßS, 17, 20βP, AD, T, E, 5α-pregnen 17α-ol-3, 20-dione, 5ß-pregnen-3α-ol-20-one and other polar steroids (triols, tetrols). The formation of the 11-oxoandrogen, 11ß-OHA, as the primary metabolite of P_4 (along with 17-OHP, T, 17, 20ßP, E_1, E_2, B and DOC) indicates the presence of at least two enzyme systems in the follicles of *C. watsoni*, cytochrome $P450_{C17}$ side chain cleavage ($P450_{C17}$) and 11ß-hydroxylase ($P450_{C11}$). $P450_{C17}$ has two enzymatic actions: 17α-hydroxylase, which convert P_4 to 17-OHP and C17-20 lyase (also called 17,20 desmolase) which is responsible for the side chain cleavage of 17-OHP to AD .The enzyme 11ß-hydroxlase ($P450_{C11}$) ultimately forms 11-oxoandrogens from AD. AD is converted to T, while E_1 is converted to E_2 by 17ß-HSD or 17-ketosteroid reductase. The enzyme P450arom is also active and converts the androgen to estrogen by 2-hydroxylation at the C-19 methyl group and consequent aromatization of the A ring of the steroid. The high level of testosterone compared to 11ß-OHA suggests greater activity of 17ß-HSD in this system. The formation of DOC from P_4 is likely to involve a 2-step enzymatic conversion of 17-OHP to DOC by 17α - hydroxylase and 21-hydroxylase (goldfish: Kime *et al.*, 1992; Arctic charr: Khan, 1996). The production of B from P_4, on the other hand, may result from 11-deoxycorticosterone (DOC) pathway (Lieberman and Prasad, 1990). The formation of 17, 20ßP as a trace metabolite of P_4 reflects secondary metabolism of 17-OHP; the enzyme 17α-hydroxylase converts P_4 to 17-OHP, which is then converted to 17, 20ßP by 20ß-HSD. The fact that 17, 20ßP was a minor metabolite of P_4 but a major product following incubation with 17-OHP can be explained on the basis of a one step enzymatic conversion by 20ß-HSD (17-OHP to 17, 20ßP). The conversion of P_4, on the other hand, would involve a 2-step process; P_4 to 17-OHP by 17α-hydroxylase followed by 17-OHP to 17, 20βP by 20β-HSD.

17, 20ßP, 11ß-OHA, B and DOC were identified as the major metabolites of 17-OHP, although the production of E_2, AD and T was also enhanced to some extent. It appears that 17-OHP favours the formation of 17, 20ßP over that of AD. The preferred synthesis of 17, 20βP from 17-OHP indicates a dominant role of 20ß-HSD in this incubation system as well as release of its sulphated conjugates. The result also affirms

the finding that 17, 20ßP plays a major role in oocyte maturation in *C. watsoni*. Priming the oocytes with hCG resulted in enhanced conversion of 17-OHP (only ~10% substrate left unmetabolized) after 72hrs. Enigmatically, the production of 17, 20βP was curtailed. Instead, several unknown steroids appeared in the medium in substantial quantities, in addition to their corresponding sulphated conjugates. It would be interesting to identify these unknown metabolites in future *in vitro* investigations on this species, especially in view that several other progestogens (triols and tetrols) are synthesized in the follicles of some teleosts (Kime, 1993).

Incubation of the ovarian follicle in the presence of exogeneous 17, 20ßP resulted in its conversion principally to a variety of unknown free and conjugated metabolites. In hCG-primed follicles, the metabolism of 17, 20βP was further enhanced. Not only did the conjugated 17, 20βP metabolites increase in quantity but also the release of unknown metabolites was restricted to only a few of these. In several other species, such other steroids as 17, 20β,21-P (17,20β, 21-trihydroxy-4-pregnen-3-one), 17,20β-P5 (5-pregnen-3β, 17,20β-P5) and 11-deoxycortisol (17,21-dihydroxy-4-pregnen-3, 20-dione; 17, 21P) have been found to be as effective *in vitro* in causing oocyte maturation as does 17, 20βP (Kime, 1993). Some of the metabolites of P_4 also did not elute with the reference steroids in the present investigation. Lack of appropriate steroids and/or lack of absorbance of some of the reduced progestogens at 254 nm makes it difficult to identify all of the metabolites. It is possible that some of these unknown metabolites are pregnane triols and tetrols, since Kime *et al.* (1991) have shown that these polar 5α-reduced metabolites of P_4 are produced by fish ovaries. Similarly, Venkatesh *et al.* (1992b) have found that gestational follicles of guppy also convert P_4 to 5α-reduced and 7-hydroxylated steroids. The present observations on the recovery of metabolites of the progestins *in vitro*, thus, necessitate future investigations to identify the unknown metabolites that were detectable particularly following incubations with P_4, 17-OHP and 17, 20βP. This would also be valuable in throwing further light on the extent to which 17-OHP and 17, 20βP can be deemed to be the final MIS in *C. watsoni* and/or *B. vagra*. Kime (1990) and Kime *et al.* (1991) have shown that exogeneous 17-OHP and 17, 20ßP are rapidly converted into very polar reduced metabolites during final oocyte maturation (see also Levavi-

Zermonsky and Yaron, 1986; Epler *et al.*, 1987). Notwithstanding the overwhelming support to the status of 17, 20βP as the MIS, other works have shown that this progestin is of lesser significance in some species of fish. It has not been readily detectable *in vivo* or *in vitro*, at least in sufficient amounts, in some species (Kime, 1990; Mugnier *et al.*, 1997; Howell and Scott, 1989). It has been thought in these instances that 17, 20βP is perhaps rapidly metabolized (*C. carpio*: Kime, 1990). In *C. carpio*, metabolites resembling 5β-pregnane-3α, 6α, 17, 20β and 20α -tetrols seem to be the final products of P_4 and 17-OHP. A metabolite resembling 20ά-hydroxy pregnane is formed in incubations of oocytes with 17, 20βP (Kime, 1990). Thus, it seems that the salmonid model may not necessarily be applicable to the cyprinids without further research. In perciform species (*Micropogonias nebulosus* and *Cynoscion nebulosus*), 20βS (17α, 20β, 21-trihydroxy-4-pregnen-3-one) has been reported to be equipotent with 17, 20βP *in vitro* (Trant *et al.*, 1986; Thomas and Trant, 1989; Trant and Thomas, 1989a, b; Patino and Thomas, 1990b, c; Thomas, 1994). In two flatfishes (*Limanda limanda* and *Pleuronectes platessa*) both are equipotent as MIS *in vitro* (Canario and Scott, 1987a, c, 1990b) but these are not synthesized *in vitro* in these species. Instead, 20ά-reduced steroids (17, 20ά-dihydroxy-4-pregnan-3-one (17, 20α -P) and 3β, 17, 20α -trihydroxy-5β-pregnane (17, 20α -P-5β)) are synthesized (Canario and Scott, 1987a, b, 1989b) and also exist in the plasma following injections of hCG. In *Morone americana* and *Morone chrysops* also, 20βS is equipotent with 17, 20βP (King *et al.*, 1995) as well as in several other species (Trant *et al.*, 1986; Thomas and Trant, 1989; Trant and Thomas, 1989b; Patino and Thomas, 1990b, c).

Conjugated steroids

Steroids are readily excreted in conjugated form owing to their greater water solubility compared to free steroids. Conjugated steroids have attracted growing interest particularly in the light of evidence of their possible pheromonal role in fish reproduction (*C. carpio*: Colombo *et al.*, 1980, 1982; *Clarias gariepinus*: Schoonen *et al.*, 1987; Van den Hurk *et al.*, 1987; Scott and Turner, 1991; Kime *et al.*, 1993; *Clarias lazera*: Van den Hurk *et al.*, 1987). Van den Hurk and Lambert (1983), Lambert *et al.* (1986), Scott and

Canario (1987), Canario and Scott (1989a, 1990a, b) and Kime (1990) have suggested that conjugated steroids as well as high levels of MIS and MIS-related metabolites in the blood play a pheromonal role. It has also been suggested that these metabolites protect the next batch of oocytes from premature exposure to maturation-inducing steroids (Kime, 1990). The gonads are major site of glucuronation and sulfation of reproductive steroids in teleosts (Scott and Vermeirssen, 1994). Glucuronyl and sulfuronyl transferases that are responsible for esterification of the hydroxyl group of steroids have been detected in the ovaries and testes of a number of fish species (Scott and Vermeirssen, 1994). In the present investigation, conjugated steroids were studied in the ovarian fragments of *C. watsoni* and *B. vagra* in March. April and May (vitellogenic to spawning period). Both glucuronated and sulphated forms were detectable in the two species. while the two species showed slight differences in proportion and periodicity of glucuronation and sulphation, of relatively greater interest is the markedly enhanced conjugation of the substrates in ovarian samples of fish that had already entered the spawning phase (April/May). Both glucuronidated and sulphated steroid metabolites were detectable in high quantities in the incubation medium following exposure of the follicles of *C. watsoni* to various steroidal substrates. This clearly indicates the presence of glucuronyl and sulpuronyl transferases in the ovarian follicles of this species, which actively metabolize the estrogens, androgens and progestins. Future investigations on conjugated steroids in these species would be valuable in determining their precise role in reproduction, if any.

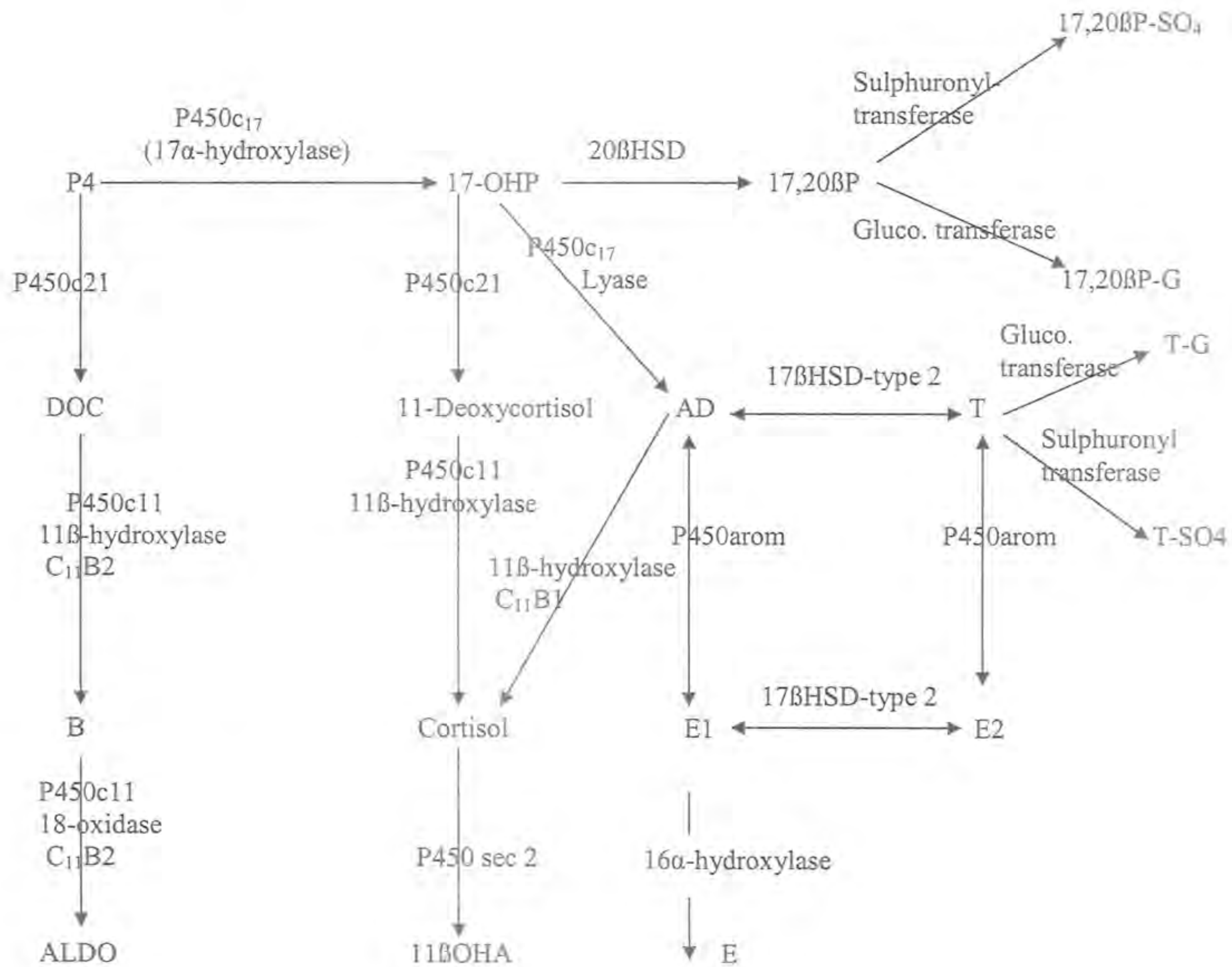

Fig.24. Scheme of synthesis of steroids based on the data presented for the ovaries of *Barilius vagra* and *Cyprinion watsoni*.

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