GREAT LAKES FISHERY COMMISSION

1992 Project Completion Report[1]

Vertebrate Sex Determination/Differentiation Workshop

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VERTEBRATE SEX DETERMINATION/DIFFERENTIATION WORKSHOP

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(Report to Great Lakes Fishery Commission)

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ABSTRACT

Sea lamprey (Petromyzon marinus) populations in the Great Lakes are being controlled primarily by applying selective lampricides to sea lamprey producing tributaries. Because of an increasing concern about the use of toxicants in the environment, the Great Lakes Fishery Commission is looking for other control methods that will reduce or eliminate the dependency on lampricides. Experts on sex determination and differentiation in mammals, amphibians, birds, and fishes met with personnel from the sea lamprey control program and presented the latest information and techniques available in their fields. Discussions were held on what directions sea lamprey research might go in order to better understand sex determination and differentiation in lampreys and a list of research needs was developed. The discussions focused primarily on sterilization methods (for spawning-run adults, embryos, prolarvae, and larvae), sex reversal techniques, and metamorphosis but also included other research needs. Specific areas of research were listed and prioritized and suggestions were made on ways to facilitate research of this type.

INTRODUCTION

The extraordinary amount of damage to the fishery of the Great Lakes caused by the invasion of the sea lamprey (Petromyzon marinus) has resulted in one of the largest and most intensive efforts to control a vertebrate predator ever attempted. The use of selective toxicants to kill larval lampreys in streams has successfully controlled lamprey populations in the Great Lakes. However, there is increasing concern about the continued use of chemicals in the environment and the Great Lakes Fishery Commission, which directs the sea lamprey control program, is looking for other control methods which will reduce or eliminate our dependency on lampricides as a control method. Scientists in other disciplines are being encouraged to participate in a series of workshops designed to encourage fresh approaches and ideas. This report summarizes the discussion and ideas generated at one of these workshops.

PURPOSE AND ORGANIZATION OF WORKSHOP

It is the Great Lakes Fishery Commission's desire to bring experts from other disciplines together with sea lamprey personnel in an attempt to stimulate new thoughts that will result in imaginative initiatives and renewed enthusiasm in the sea lamprey control program. With this in mind, experts on sex determination and differentiation in mammals, amphibians, birds and fishes were brought together with sea lamprey personnel to discuss the latest information and techniques available in their fields. Each made a short presentation describing their research, after which there was a question and answer period. After all reports were presented, discussions were held on what directions sea lamprey research might go in order to better understand sex determination and differentiation in sea lampreys. A list of research needs were identified that may be useful in improving the sea lamprey control program and reducing dependency on selective toxicants.

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PRESENTATIONS (SUMMARIES AND ABSTRACTS)

The Great Lakes Fishery Commission and its Goals (Roger Bergstedt)

Mr. Bergstedt described the sea lamprey control program and showed the slide show entitled "The Sea Lamprey: Great Lakes Invader." The structure of the Great Lakes Fishery Commission and its subgroups was described.

Summary of Life History and Current Knowledge (or lack of) on Sex Determination and Differentiation in the Sea Lamprey (Stacia Sower)

Dr. Sower summarized the life history of the sea lamprey and described the development of the gonads. The structure, distribution in the brain, and biological activity of lamprey GNRH was described. The spawning behavior and plasma steroid levels present in lampreys during specific spawning behavior was

presented. The potential for using a lamprey GNRH antagonist as a lamprey sterilant was discussed and preliminary study results were described.

Sex Differentiation in Teleosts and Cyclostomes and Possible Strategies for Reproductive Control of Lampreys (Aubrey Gorbman)

A century of study of teleostean gonadal sex differentiation has produced surprisingly few generalizations. Among the teleosts there are so many patterns of gonadal differentiation, organization and genetic relationships that it seems clear in this group that evolution of the gonad and its regulation has undergone rapid and varied directions (Chan and Yeung, 1983). Sex chromosomes in bony fishes are, as a rule, undifferentiated morphologically. In some teleost species the males are heterogametic and in others it is the females. The teleostean gonad may develop, according to species, in a progynous or protandrous sequence, or it may be sequentially or permanently hermaphroditic. Teleostean gonadal phenotypic differentiation in many tested species can be altered by so many natural environmental or experimentally applied agents (e.g. temperature, photoperiod, population density or structure, hormones, etc.) that it would seem that a variety of autosomal regulatory genes must be influencing the process (see reviews in Chan and Yeung, 1983; Hunter and Donaldson, 1983).

Particularly interesting examples may be seen in the reversals of sex that occur in some species over time or that follow changes in social influences. In several progynous species that form small social groups, all individuals remain female except one that differentiates as a male from within the group (Shapiro, 1981). If this male is experimentally removed, a new male differentiates from among the females. Young specimens in populations of black sea bass (Centropristes) begin with a female:male ratio of 5.5:1. By age 7 the ratio is 0.04:1, and by age year 10 all specimens are males (Cochrane and Grier, 1991). Temperature during development has been found influential in altering population sex ratios in some fish species (Chan and Yeung, 1983). Numerous experiments have been done with androgenic, estrogenic and other hormones to alter the phenotypic sex from that expected of the genotype of a variety of teleostean species (Chan and Yeung, 1983). A practical use of this approach is the production of unisexual, or even sterile, populations of salmonid species (Hunter and Donaldson, 1983; Yamazaki, 1983).

A surprising aspect of this field is that the histological and cytological features of normal and altered sex differentiation have rarely been examined. In most instances results of experimental manipulation have been judged by later visual inspection of the gonads of partly or fully grown individuals.

With this curtailed summary of information from the teleosts, the present discussion will focus on sex differentiation in the cyclostomes, a topic that has received very little attention. Early work by Okkelberg (1914, 1921) and D'Ancona (1943, 1950) claimed that the brook lamprey (Entosphenus) and anadromous lampreys (Petromyzon) are protandrous. They interpreted the small solid cystic structures in the early gonads of ammocoetes to be male in nature. However, in the only definitive studies of sex differentiation in Petromyzon marinus, Hardisty (1965, 1971) showed convincingly that these single or grouped cells become oocytes in all individuals and, therefore, this lamprey is in fact progynous. For the entire ammocoete stage (a period of years) these oocytes merely proliferate mitotically and enter meiosis in increasing numbers. Meiosis during this long period is arrested in the prophase (diplotene-dictyate) stage. At the time of metamorphosis, in animals destined to become males, the oocytes become atretic. At that time a new generation of nests of spermatic elements develops from persisting stem cells. In definitive females the first generation oocytes remain and enter a vitellogenic phase.

Ideas about sex differentiation in the myxinoids (hagfish) had a similar evolution. Earlier students of the phenomenon (Cunningham, 1886; Nansen, 1887) committed the same error, in judging the earliest gonadal elements to be male. Later investigations (Schreiner, 1955; Gorbman, 1990) were able to show that the first small cystic structures that form in the gonad eventually become groups of oocytes. Thus, the hagfish, like the lampreys, are progynous.

For neither the hagfish or the lampreys has there been any significant study of factors that may alter sex differentiation, as they may in teleosts. That there may be susceptibility to environmental regulators of gonadal differentiation was suggested by Gorbman and Dickhoff (1978) who found regional differences in sex ratios in different populations of the hagfish Eptatretus stouti. These ratios are always in favor of female differentiation, varying from 1:1 to 3:1. To my knowledge there has been no similar comparison of sex ratios in populations of lampreys.

Hardisty and Taylor (1965) treated ammocoete larvae with sex steroids and, in the only such study, found no effect on sex differentiation. However, these experiments can hardly be considered definitive, and they need to be repeated by use of a broader protocol and testing of timing and dosage of hormone administration, as well as choice of steroids.

At this time it would appear that manipulation of reproductive function may offer one of the most attractive alternatives to use of lampricides in control of numbers of

parasitic lampreys like Petromyzon marinus. The major disadvantage of current or proposed systems of lamprey control is that they must be applied indiscriminately to natural environmental media. Whether these measures involve toxic lampricides or agents that might compromise metamorphosis or adult reproduction, they incur a certain degree of hazard to other species, including humans. If early lamprey larvae are as susceptible to hormonal modification or sterilization of the gonad as are the teleosts, then an environmentally benign alternative suggests itself. It is possible to obtain large numbers of laboratory-fertilized eggs and/or early larvae for hormonal treatment. If an appropriate timing and dosage protocol can be worked out then agonadal sterile ammocoetes can be produced for release into spawning streams.

The introduction of sterile males into the breeding environment of sea lampreys is already an active field of investigation, particularly at Hammond Bay. It appears to have promise in reducing reproductive efficiency of the natural population of sea lampreys. In practice it involves the capture of anadromous mature males, their injection with a chemical sterilant (bisazir) followed by placement in spawning streams. While this procedure has promise, it produces relatively few sterile males (several thousand) per run at this time. Hormonal sterilization of early ammocoetes could produce thousands of sterile animals of both sexes from a single clutch of eggs from one female. Handling of millions of eggs and yolked post-hatching lamprey larvae requires little in the way of manpower or facilities, and it approaches a different phase of the life cycle. Sterile ammocoetes, by their sheer number, would compete with the developing normal ammocoete population, and later with the normal anadromous adults.

Another strategy that has not been explored is the identification of the sex pheromone(s) utilized by spawning lampreys to attract reproductive partners or to evoke other reproductive behaviors. Use of such pheromones in spawning streams could confuse reproductive pairing, or it could be the basis for a program of trapping. This, too, is a current research topic, with several investigators trying to identify attraction pheromones, or other types of attractants. It appears that much of this current work is as yet unpublished. Attention appears to be directed to finding pheromones of a peptide nature. I would like to suggest that more emphasis should be given to steroids and extracts of ripe gonads. Modified gonadal steroids already have been identified as pheromonal sex attractants in several teleostean species. The most effective use of pheromonal attractants probably would be in conjunction with trapping programs. According to Bergstedt's recent summary for this meeting, traps, even when unbaited, have a significant degree of success in lamprey control, especially when used with physical barriers. This effectiveness probably could be made even greater

by use of pheromonal baits, even without supplementation by expensive physical barriers.

A third approach is suggested by the recent finding that in all vertebrates studied so far, gonadotropin-releasing hormone (GnRH) secreting cells arise in the early embryonic olfactory epithelium. They must migrate from this epithelium into the hypothalamus to be effective eventually in reproductive regulation (Schwanzel-Fukuda and Pfaff, 1989; Schwanzel-Fukuda et al., 1989; Murakami et al., 1991). If a procedure can be devised to interfere with differentiation of the olfactory epithelium and formation of GnRH cells, another method for producing sterile lampreys could be made available. The essential role of GnRH in reproduction of lampreys has been reviewed by Sower (1990).

Interference with reproduction by a method of direct exposure of lamprey eggs and/or larvae to agents in the laboratory offers the possibility of eliminating any possible environmental hazard. The first priority in starting such a research program is to determine sex ratios in adult lamprey populations. This would indicate whether lamprey sex differentiation is possibly susceptible to epigenetic factors. At the same time procedures for developing supplies of sterile animals by hormonal or other means should be devised. In addition, efforts to identify sex pheromones should be investigated. It would appear at this time that direct treatment of eggs and early lamprey larvae has promise for meeting environmental requirements.

Literature Cited

Chan, S. T. H., and W. S. B. Yeung, 1983. Sex control and sex reversal in fish under natural conditions in Fish Physiology, Vol 9B (W. S. Hoar, D. J. Randall, E. M. Donaldson, editors). Academic Press, Orlando, pp. 171-222.

Cochran, R. E., and H. J. Grier, 1991. Regulation of sexual succession in the protogynous black sea bass, Centropristis striatus (Ostecichthyes, Serranidae). Gen. Comp. Endo., 82:69-77.

Cunningham, J. T., 1886. On the structure and development of the reproductive elements in Myxine glutinosa. Quart. J. Microbiol. Sci., 27:49-76.

D'Ancona, U., 1943. Nuova ricerche sulla determinazione sessuale dell' anguilla. Arch. Oceanogr. Limnol., 3:159-271.

D'Ancona, U., 1950. Determination et differenciation du sexe chez les poissons. Arch. Anat. Micr. Morph. Exp., 38:174-294.

Gorbman, A., 1990. Sex differentiation in the hagfish Eptatretus stouti. Gen. Comp. Endo., 77:309-323.

Gorbman, A., and W. Dickhoff, 1978. Endocrine control of reproduction in the hagfish. in Comparative Endocrinology (P. Gaillard and H. Boer, editors) Elsevier, Amsterdam.

Hardisty, M. W., 1965. Sex differentiation and gonadogenesis in lampreys. II. The ammocoete gonads of the landlocked sea lamprey Petromyzon marinus. J. Zool. (London) 146:346-387.

Hardisty, M. W. 1971. Gonadogenesis, sex differentiation and gametogenesis. in The Biology of Lampreys (M. W. Hardisty and I. C. Potter, editors) Academic Press, New York.

Hardisty, M. W. and E. J. R. Taylor, 1965. The effects of sex hormones on the ammocoetes larva. Life Sci., 4:743-747.

Hunter, G. A., and E. M. Donaldson, 1983. Hormonal sex control and its application to fish culture. in Fish Physiology, Vol 9B (W. S. Hoar, D. J. Randall, and E. M. Donaldson, editors). Academic Press, Orlando.

Murakami, S., T. Seki, K. Wakabayashi, and Y. Arai, 1991. The ontogeny of luteinizing hormone-releasing hormone (LHRH) producing neurons in the chick embryo: possible evidence for migrating LHRH neurons from the olfactory epithelium expressing a highly polysialated neural cell adhesion molecule. Neurosci. Res., 12:421-431.

Nansen, F., 1887. A protandric hermaphrodite (Myxine glutinosa) amongst the vertebrates. Bergens Mus. Aareberetning, 7:3-39.

Okkelberg, P., 1914. Hermaphroditism in the brook lamprey. Science, 39:478-479.

Okkelberg, P., 1921. The early history of the germ cells in the brook lamprey Entosphenus wilderi (Gage) up to and including the period of sex differentiation. J. Morph., 35:1-151.

Schreiner, K. E., 1955. Studies on the gonad of Myxine glutinosa L., Universitet i Bergen, Arbok 1955, Naturvit. Rekke, No. 8. 45 pages, 4 plates.

Schwanzel-Fukuda, M., D. Bick, and D. W. Pfaff, 1989. Luteinizing hormone-releasing hormone (LHRH) expressing cells do not migrate normally in an inherited hypogonadal (Kallman) syndrome. Mol. Brain Res., 6:311-326.

Schwanzel-Fukuda, M. and D. W. Pfaff, 1989. Origin of luteinizing hormone-releasing hormone neurons. Nature, 338:161-164.

Shapiro, D. Y., 1981. Size, maturation and social control of sex reversal in the coral reef fish Anthias squamipinnis (Peters). J. Zool., 195:128.

Sower, S. A., 1990. Neuroendocrine control of reproduction in lampreys. Fish Physiol. Biochem., 8:365-374.

Yamazaki, F., 1983. Sex control and manipulation in fish. Aquaculture, 33:329-354.

Labile Sex Determination in Lampreys (Margaret F. Docker)

In birds and mammals, genotypic sex determination is overwhelmingly the rule. In contrast, although there is little doubt that sex also has a genetic basis in fish, amphibians, and reptiles, the expression of sex in these so-called lower vertebrates is variable and often quite labile (Yamazaki 1983). Many of the lower vertebrates, for example, appear to lack

heteromorphic sex chromosomes (Bull 1980; Yamazaki 1983), and environmental factors can alter sex differentiation in a number of species. Incubation temperature, for example, has been shown to influence sex determination in many turtles, an alligator, and a teleost fish (e.g., Bull 1980; Conover 1984). The bisexuality of the hagfish gonad and a prolonged stage of sexual indeterminacy in lampreys (Hardisty 1985) suggest that sex differentiation in cyclostomes may also be labile. Furthermore, in lampreys, adult sex ratios have often been related to their abundance. In particular, among the landlocked sea lamprey, Petromyzon marinus, of the upper Great Lakes, the sex ratios of both adults and larvae varied widely with abundance (Purvis 1979).

Given this relationship between abundance and sex ratio in the upper Great Lakes following treatment of streams with the lampricide TFM, I wished to evaluate the effect of density on lamprey sex ratios under experimental conditions. Sea lamprey larvae from a tributary to Lake Champlain, which had not been treated with TFM, were maintained in outdoor tanks at ambient temperatures for over 3 years. Experimental densities were ca. 10, 20, 50, and 100 larvae per 0.3-m^2 tank, but actual number of larvae per m^2 ranged from 57 to 470 initially and from 23 to 213 after the 3 years. As in the upper Great Lakes, sex ratio (% male) of previously undifferentiated larvae (i.e. those <60 mm initial length) increased marginally with density ($P = 0.06$). It was not possible to eliminate mortality (which exceeded 60%), and thereby the possibility of differential mortality between the sexes, but among the dead larvae that were recovered, there was no evidence of sex-specific mortality. The sex ratio of larger larvae ($\geq$60 mm initial length) was unaltered by density. It appears, therefore, that sex differentiation in lampreys is influenced by density, but that sometime between the onset of germ cell proliferation at ca. 60 mm and the point at which sex is discernible at ca. 90 mm, the gonad loses its lability with respect to density.

A similar relationship between sex ratio and density was investigated in the nonparasitic least brook lamprey, Lampetra aepyptera, which also has never been subjected to TFM. In this field study, larvae were electrofished from 12 stream in MD, DE, KY, TN, and AL. Sex ratios ranged among the streams from 24% to 71% male and, as in the sea lamprey, the proportion of males increased significantly with relative density (relative density among the streams was estimated from the number of larvae collected per m^2 of substrate). The skewed sex ratios were not likely due to differential mortality between the sexes: the differences in sex ratio among streams were established at the time of gonadal differentiation and remained relatively constant thereafter. Furthermore, although females predominated in the oldest larval age class, thus appearing to metamorphose later than males, such differential recruitment to the adult population

had little effect on the overall sex ratio. Density-dependent sex determination was consequently proposed in the least brook lamprey also.

The apparent lability of sex differentiation in lampreys was further investigated through attempts at hormonal sex reversal. For 21 weeks, sea lamprey larvae were immersed in 0.01, 0.1, or 1.0 mg/L estradiol, testosterone, or methyltestosterone, and then maintained without further treatment for another 2 years until they were large enough to sex. These gonadal steroids, however, were ineffective in altering the sex ratio of either previously undifferentiated larvae (<60 mm initial length), or of larvae $\geq$ 60 mm in length. Many of the larger larvae, however, exhibited gonadal abnormalities. In particular, estradiol caused oocyte degeneration and a proliferation of stromal tissue, whereas testosterone appeared to have a general proliferative effect on both undifferentiated germ cells and oocytes. Methyltestosterone had little effect on gonadal histology, but larvae at the 2 higher dosages suffered virtually 100% mortality. Further refinement of the treatment protocol, particularly a reduction of treatment-related mortality and an extension of the treatment period, appears to be necessary. Successful hormonal sex reversal in lampreys would be a valuable experimental tool for further investigations into lamprey sex differentiation. For example, sex ratios of progeny produced by mating sex-reversed and normal individuals could determine which, if either, sex is heterogametic. Furthermore, the monosex stocks so-produced could be used to investigate sex-specific differences in gonadal morphology or steroids prior to recognizable sex differentiation.

Literature Cited

Bull, J. J. 1980. Sex determination in reptiles. Quart. Rev. Biol. 55:3-21.

Conover, D. O. 1984. Adaptive significance of temperature-dependent sex determination in a fish. Amer. Nat. 123:297-313.

Hardisty, M. W. 1965. Sex determination and gonadogenesis in lampreys. II. The ammocoete gonads of the landlocked sea lamprey, *Petromyzon marinus*. J. Zool., Lond. 146:346-387.

Purvis, H. A. 1979. Variation in growth, age at transformation, and sex ratio of sea lampreys re-established in chemically treated tributaries of the Upper Great Lakes. Great Lakes Fish. Comm. Tech. Rep. 35.

Yamazaki, F. 1983. Sex control and manipulation in fish. Aquaculture 33:329-354.

Metamorphosis of Lampreys (John H. Youson)

A particular focus has been the morphological and physiological changes associated with one of the unique phases of the lamprey life cycle, metamorphosis, when the larvae transform

into adults. Seven stages of metamorphosis have been described for the sea lamprey, Petromyzon marinus. This phase is marked by a number of developmental phenomena, including complete regression of some organs, transformation of others, and the development of still others from anlagen which have shown arrested differentiation since embryogenesis.

One of the aspects of lamprey metamorphosis which has intrigued us is that it seems to be a necessary step for the perpetuation of the species, for it is only near the completion of, or immediately after, this event that an important growth phase of the gonads is initiated. No neoteny or protandry exists in lampreys. In nonparasitic species of lampreys gonadal growth and sexual maturation is initiated immediately after the completion of metamorphosis, where in many parasitic species, such as P. marinus, there is some slow gonadal growth during the feeding interval but final sexual maturation does not begin until feeding ceases (Fig.1). In fact, feeding ceases in P. marinus or is never initiated in nonparasitic species because one of the consequences of final maturation of the gonads is the high levels of gonadal steroids at this time results in atrophy of the gut and other changes to the digestive system. Therefore, one approach to lamprey control in the Great Lakes may be to find a mechanism to stimulate an early, final sexual maturation of P. marinus before they have had time to feed. That time is during their metamorphosis.

Metamorphosis in P. marinus lasts about 4 months but animals are nontrophic for between 6-11 months. They are only able to survive this period of fasting because the larvae (ammocoetes) which enter metamorphosis have extensive lipid reserves. Despite these reserves, most animals which complete metamorphosis are emaciated and have started protein catabolism. Feeding, therefore, is an essential step prior to the commencement of sexual maturation. If sexual maturation was initiated prior to feeding, the animals would have no energy reserves to continue fasting (a consequence of gonadal maturation) and the animals would die (refer to the broken line in Fig. 1). Although it has not been tested, it is also highly unlikely that any animals that could survive this precocity and extended period of starvation would have viable gametes. Before we can initiate any programme to stimulate precocity in P. marinus, we need to know more about the factors which control gonadal growth and also what influence alterations in the events or the timing of metamorphosis have on the life history of this organism. In recent years we have been examining both of these parameters.

While examining the life history of adults of a nonparasitic species Lampetra richardsoni of lamprey on Vancouver Island, British Columbia (Youson and Beamish, Can. J. Zool. 69:628-637, 1991) we noted that a portion of one population did not develop

secondary sex characteristics at the expected time and when they were brought to the laboratory and subjected to host fish, they fed. Histological examination of these individuals, called Lampetra richardsoni: variety marifuga, showed that most were males, that their gut was not atrophied and was full of ingested host flesh, and their gonads were not mature. Electrophoretic examination of several gene loci demonstrated that the nonparasitic and parasitic varieties were the same species. Therefore, two life history types are present within the same species. Subsequent histological examination and observation has permitted us to conclude that the potential for parasitic feeding behavior has been brought about by a delay in the initiation of sexual maturation. This delay resulted in the retention of the gut and a viable digestive system and is a consequence of a retarded metamorphosis The universality of this phenomenon among lamprey population is not known but large, prespawning, (supposed) nonparasitic lampreys have been found in the watersheds of the Great Lakes. This example serves to illustrate the fact that the timing of metamorphosis-related gonadal growth is more than just of passing interest to a discussion of lamprey control. Also we can gain much useful information on sexual maturation of lampreys by establishing the reason for the earlier maturation in nonparasitic species.

A second approach has been to examine factors which might control gonadal activity, either directly or indirectly, during metamorphosis. Gonadotropin has not been isolated from lampreys but gonadotropin-releasing hormone (GnRH) has been isolated from brains of adult lampreys. Following the sequencing of this hormone, an antibody and an RIA were produced. In order to find the time that this hormone might influence the gonads, GnRH was extracted from the brains taken from P. marinus at each of the seven stages of metamorphosis and the concentration was measured by RIA (Youson and Sower, J. Exp. Zool. 259:399-404, 1991). The first GnRH to appear in significant and consistent quantity at stages 4 and 5 was not lamprey GnRH but a second GnRH. The nature of this GnRH is presently under investigation (Sower). From stages 6 to 7, the concentration of both lamprey GnRH and the second GnRH increased dramatically but the former became the dominant type. Whether GnRH has a direct influence on the activity of the lamprey gonad at this time in the life cycle has not been demonstrated. This is a subject for future study. However, since there is a relationship between brain GnRH and gametogenesis in adult lampreys, there is a strong possibility that a similar relationship exists at metamorphosis. This study serves to further emphasize the point that metamorphosis is essential before final phases of maturation of gonads can occur. Blocking either metamorphosis or the growth of gonads at this time are important parameters for consideration in lamprey control.

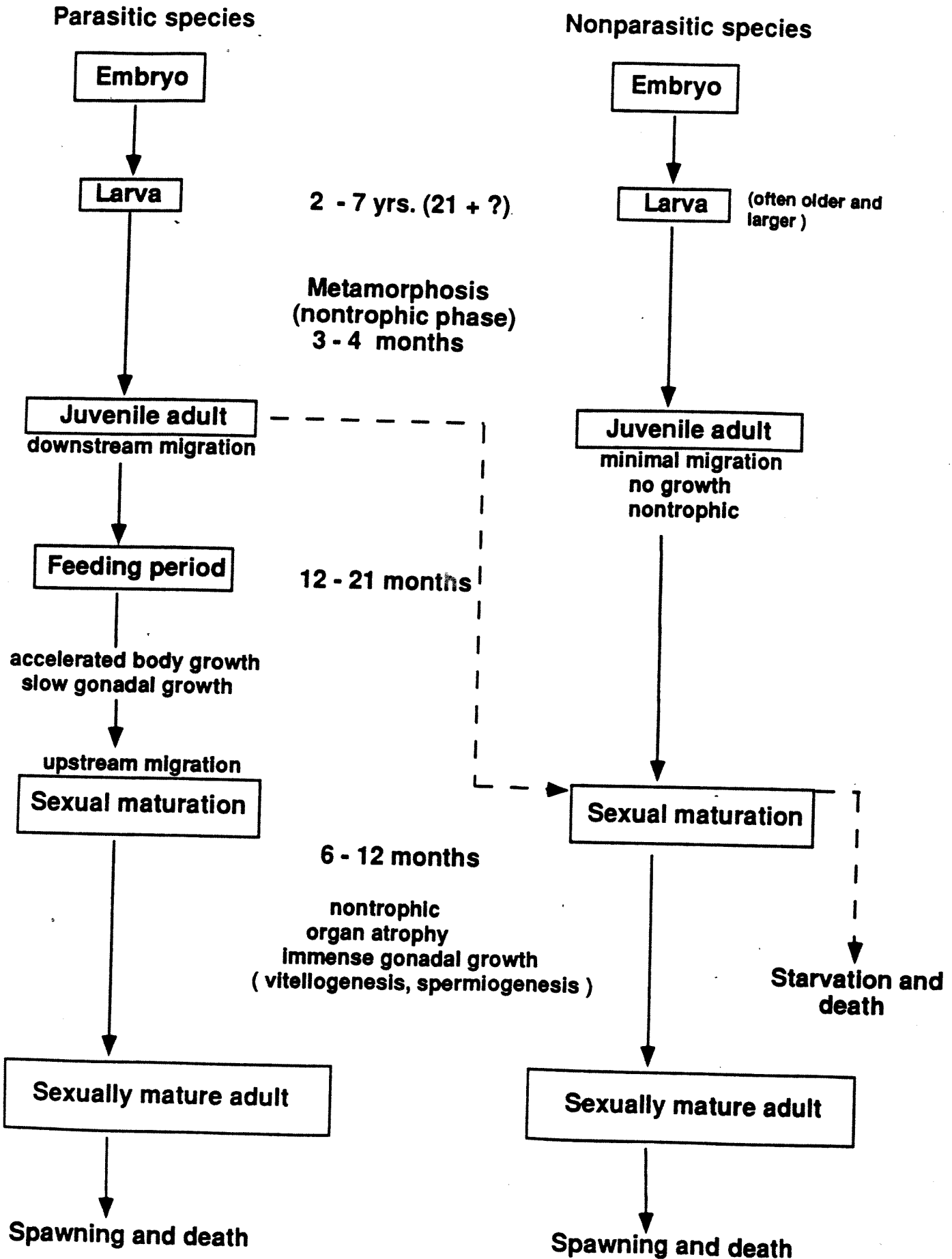

Parasitic species
Nonparasitic species
Embryo
Embryo
Larva
2 - 7 yrs. (21 + ?)
Larva
(often older and larger)
Metamorphosis
(nontrophic phase)
3 - 4 months
Juvenile adult
downstream migration
Juvenile adult
minimal migration
no growth
nontrophic
Feeding period
12 - 21 months
accelerated body growth
slow gonadal growth
upstream migration
Sexual maturation
Sexual maturation
6 - 12 months
nontrophic
organ atrophy
immense gonadal growth
(vitellogenesis, spermiogenesis)
Starvation and death
Sexually mature adult
Sexually mature adult
Spawning and death
Spawning and death

Figure 1.

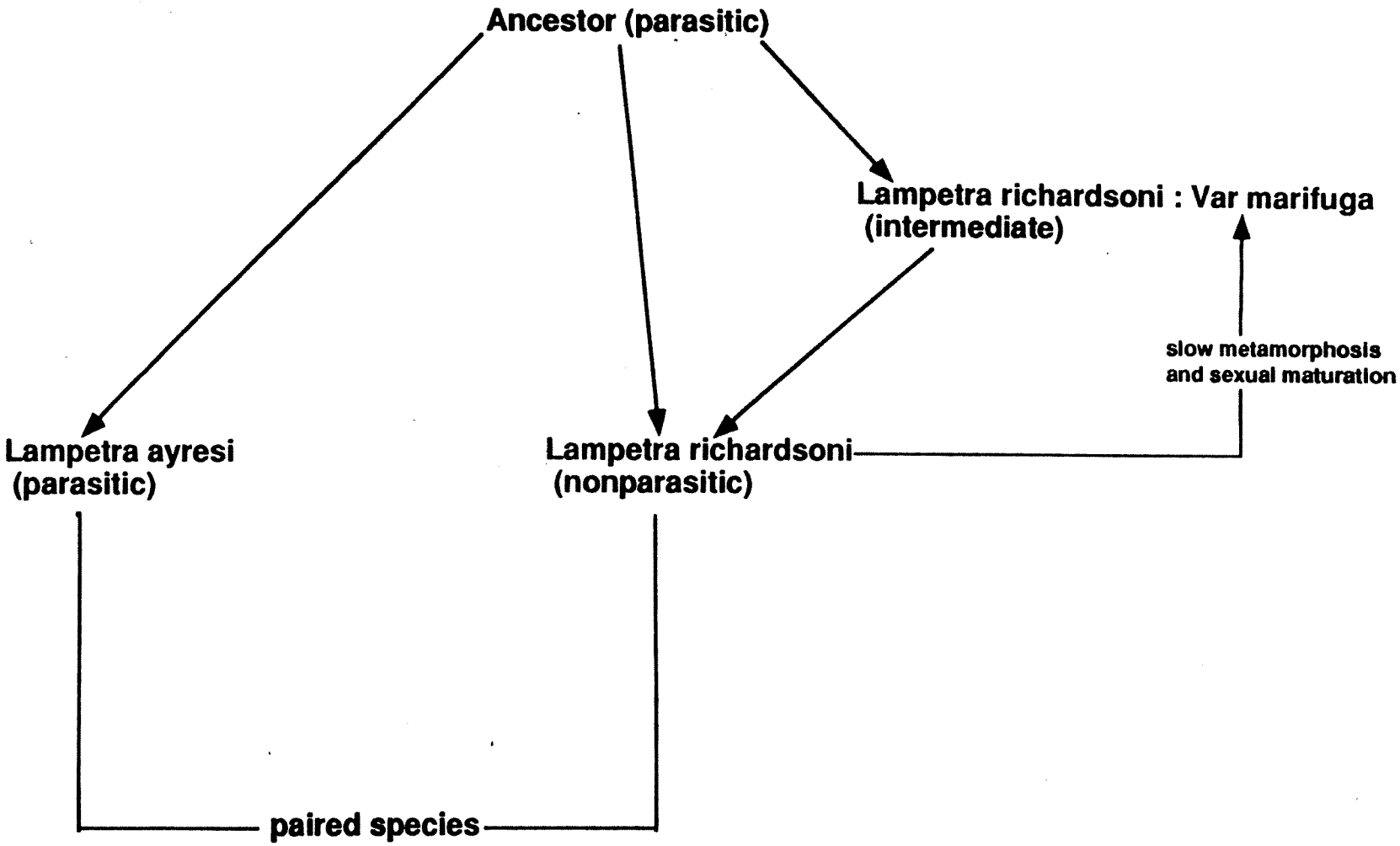


Figure 2

Research collaboration of
J. H. Youson and
R. J. Beamish

Sex Determination and Differentiation in Salmonids (Martin S. Fitzpatrick, Carl B. Schreck, G. Feist, J. M. Redding, and C. G. Yeoh)

The study of sex determination and sex differentiation in fish has important application to the field of aquaculture and offers potential insights into a general understanding of sexual development in vertebrates. The differential value of one sex over the other in specific aquaculture circumstances has spurred the development of techniques to control sexual development. Our laboratory has studied the use of two of the more common techniques: chromosome set manipulation and sex inversion through treatment with sex steroids.

We have used gynogenesis to produce all-female populations of rainbow trout (Oncorhynchus mykiss), chinook salmon (O. tshawytscha), and chum salmon (O. keta). The standard technique involves irradiation of sperm with UV light followed by fertilization of eggs and subsequent heat shock to force retention of the polar body (thereby returning the developing embryo to diploidy). Although the technique is very successful at producing all-female populations, survival is low (10-30%); therefore, we have used hormone treatment following gynogenesis as a means to produce sex-inverted gynogens (phenotypic males/genetic females) that are capable of maturing and producing sperm which will produce only female off-spring. Sperm from such fish can be cryopreserved to retain the capacity to produce all-female populations. Performance studies on gynogenetic chinook salmon suggest that they cope with stress, resist disease, and osmoregulate as well as normal fish. The production of all-male populations can be achieved through androgenesis which is similar to gynogenesis except that the eggs are irradiated, then fertilized with normal sperm, and finally heat shocked (the resulting embryos are normal females or "super males" whose off-spring will only be male).

We have studied the use of steroid treatment for causing sex inversion in rainbow trout, chinook salmon, chum salmon, and coho salmon (O. kisutch). We have found that feeding estradiol is a potent way to feminize salmon (rainbow trout do not respond to this type of treatment as well as the salmon). The synthetic androgen, 17 α-methyltestosterone, is a potent masculinizer (or sterilizer, depending on dosage and exposure time) when the fish are immersed in it around the time of hatching or fed it during the first 3 months of feeding. The naturally occurring androgen, 11β-hydroxy-androstenedione, is also a potent masculinizer, but the effects are permanent only when the fish are immersed in and fed the steroid. Testosterone, androstenedione, and cortisol had no effect on sexual development.

The potency of steroid treatment for causing sex inversion raises the question of what, if any, role do endogenous steroids

play in sex differentiation? If endogenous steroid production is involved in differentiation of the gonad, then steroids must be present prior to and during the differentiation process. Whole body steroid levels were measured in coho salmon and rainbow trout. The results indicated that steroids are present throughout development. Studies on *in vitro* production of steroids during early development corroborated the whole body studies and suggested that the difference between males and females may be first manifested in the gonadal response to pituitary stimulation of steroid production. Furthermore, interrenal production of androstenedione was pronounced very early in development--before any signs of gonadal differentiation--opening the possibility of sexualization of the gonad by steroids of non-gonadal origin.

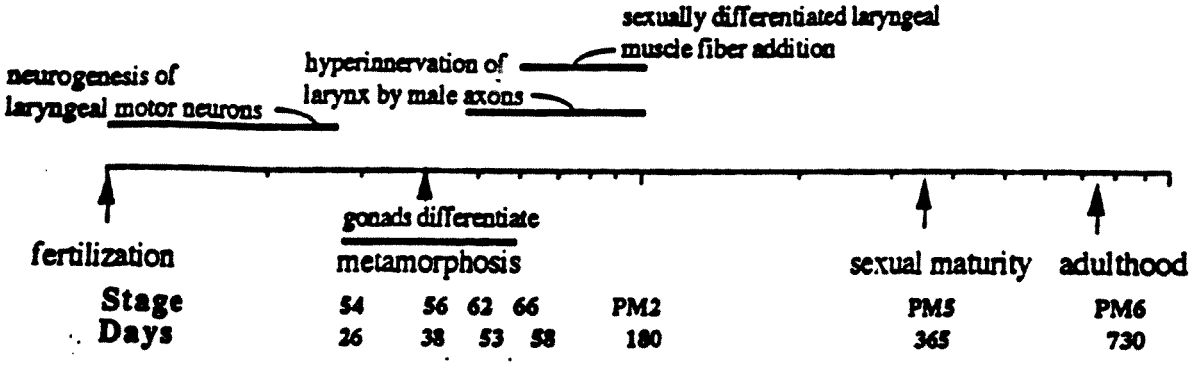
We have investigated the mechanism of steroid-induced sex inversion. Feeding steroids to rainbow trout alters the *in vitro* production of steroids from the gonads. Antibodies to sex-specific gonadal proteins from coho salmon were used in Western Blot analysis of gonadal homogenates from gynogenetic chinook salmon that had been masculinized by methyltestosterone treatment. Such analysis revealed that within 175 days of fertilization, male- and female-specific proteins could be detected in masculinized and normal females, respectively. The results indicate a link between steroid treatment and protein production which suggests that the steroids are acting through conventional mechanisms of steroid action. We have begun looking for an androgen receptor in ovarian cytosol. Using the ligand mibolerone (a synthetic steroid similar to methyltestosterone), we have found binding activity in ovarian cytosol from immature but not mature rainbow trout. Saturation binding studies indicate a high affinity, low capacity binding site. Competition studies have demonstrated that displacement potency follows the following scheme: mibolerone = methyltestosterone > dihydrotestosterone >> testosterone, 11-ketotestosterone, estradiol, 17a, 20β-dihydroxy-4-pregnen-3-one >> 11β-hydroxy-androstenedione, androstenedione. Finally, we are currently attempting to isolate unique mRNA from methyltestosterone-treated chinook salmon yolk-sac fry using subtractive hybridization techniques. These studies are on-going.

Sexual Differentiation of a Laryngeal Neuromuscular System in the Clawed Frog, Xenopus laevis; Interaction of Thyroxine and Androgenic Steroids (Darcy Kelley and John Robertson)

Brain and muscle are sexually differentiated tissues in which masculinization is controlled by the secretion of androgens from the testes. Sensitivity to androgen is conferred by the expression of an intracellular protein, the androgen receptor. A central problem of sexual differentiation is thus to understand the cellular and molecular basis of androgen action. We do not understand how hormone occupancy of a receptor translates into an

alteration in the developmental program of the target cell. Our studies on sexual differentiation of brain and muscle in Xenopus laevis are designed to explore the molecular basis of androgen induced sexual differentiation by examining how this hormone controls the masculinization of brain and muscle targets.

Our approach to this problem has focused on a highly androgen sensitive, sexually dimorphic neuromuscular system: laryngeal muscles and motor neurons of the clawed frog, Xenopus laevis. The developmental program which permits expression of the masculine phenotype in this system is controlled by androgen secretion. The larynx of Xenopus laevis is one of the most androgen sensitive tissues in any vertebrate; levels of expression of androgen receptor in developing larynx are in excess of mammalian testis or human prostatic tumors (Kelley et al., 1989; He et al., 1990). The extreme androgen sensitivity of this system and the ease of developmental studies in an amphibian make androgen-controlled development of laryngeal muscle and motor neurons a powerful experimental model system for cellular and molecular exploration of sexual differentiation.



Sexual differentiation in Xenopus laevis is summarized above. Laryngeal motor neurons are generated from tadpole stage 101/2 until 54 (Gorlick and Kelley, 1987). Their axons reach the larynx at stage 40. The gonads differentiate at tadpole stage 56 (Niewkoop and Faber, 1956). At this time, the number of axons in each laryngeal nerve is high (~650) and the same for males and females (Kelley and Dennison, 1990). In females, axon numbers *decrease* from tadpole stage 56 on, reaching lower (~225) adult values at post-metamorphic stage 2 (PM2, stages of Tobias et al., 1991a). In males, axon numbers *increase* between tadpole stages 59 and 62 (~750 axons) and then decline; adult values (~350 axons) are reached sometime after PM2. Adult male Xenopus laevis have ~350 and adult female ~250 laryngeal motor neurons as determined by retrograde labelling from laryngeal muscle; each neuron sends a single axon to the larynx (Simpson et al., 1986). Thus, sex differences in axon numbers are present from stage 59 through adulthood and are attributable to two processes: more gain in males during early tadpole development followed by less loss during late tadpole and early post-metamorphic development.

The increase in axon number observed in male tadpoles between stages 59 and 62 is controlled by androgen (Robertson et al., 1991). Axon addition is blocked by the antiandrogen hydroxyflutamide; values fall to female levels (axon number in females is unaffected). Treatment with the androgen dihydrotestosterone (DHT) increases axon numbers in both sexes.

Sexual differentiation of laryngeal muscle fiber number occurs after metamorphosis is complete (see diagram). Sexually dimorphic numbers of fibers are present at PM1; by PM2, fiber number averages 31,353 in males and 10,175 in females (Marin et al., 1990) and essentially adult numbers have. Post-metamorphic fiber addition is due to myoblast proliferation and fusion to form new fibers (Sassoon et al., 1986; Sassoon and Kelley, 1986). Sex differences in the rate of post-metamorphic muscle fiber addition are attributable to androgen secretion in males. The antiandrogen, flutamide, blocks the dramatic muscle fiber addition characteristic of juvenile males (Sassoon and Kelley, 1986). In juvenile frogs, androgen administration causes myoblasts to proliferate; newly generated myoblast nuclei are found in immature muscle fibers (Sassoon et al., 1986). More recently (Marin et al., 1990), we have shown that, within a critical developmental period, castration blocks muscle fiber addition in males and androgen administration increases muscle fiber number in females; implantation of a testis can induce masculine muscle fiber numbers in juvenile females.

The onset of sexual differentiation of the laryngeal neuromuscular system in X. laevis coincides with the onset of metamorphosis, the process during which a tadpole is transformed into a frog. Amphibian metamorphosis is controlled by the hormone thyroxine which acts via receptors that are members of the steroid hormone receptor gene super family to which the androgen receptor also belongs. The major active hormone is believed to be T_3 (thyroxine). Thyroxine levels begin to rise at tadpole stage 56, peak between stages 59 and stage 62, and then fall again to very low levels at stage 66 (Leloup and Buscaglia, 1977).

To determine if sexual differentiation is affected by thyroxine secretion we administered androgen to 6-n-propylthiouracil (PTU) treated tadpoles. PTU treatment prevents thyroxine synthesis and arrests tadpole development at stage 54; metamorphosis cannot proceed. Tadpoles were PTU or PTU plus dihydrotestosterone (DHT)-treated for 104 days beginning at stage 48. As expected, metamorphosis was blocked and the external morphology of treated animals (limb buds, tentacles etc.) did not progress beyond stage 54 although the tadpoles continued to grow in size. Unexpectedly, gonadal development was not blocked by PTU treatment. At stage 54 gonads are normally undifferentiated; testes cannot be distinguished from ovaries even with the electron microscope. After 104 days of PTU treatment, the

ovaries contained well developed oogonia and the testes contained spermatagonia.

Normally, tadpole larynges are very sensitive to exogenous androgen. A dose of 0.25 mg/l begun at stage 54 will kill all tadpoles by stage 59 through laryngeal hypertrophy interference with digestive and cardiac functioning. This response to DHT was not observed in PTU-treated tadpoles. Further, PTU treatment alone blocked laryngeal development. While some enlargement of larynges occurred in the 104 days of PTU treatment, morphological development of larynges did not progress beyond that found in untreated stage 54 tadpoles. Laryngeal axon number increased. This effect probably is due to the thyroxine sensitivity of the motor neurons rather than a response to androgen because PTU blockade is known to maintain elevated axon numbers in Xenopus oculomotor neurons (Schonenberger and Escher, 1988) which do not express androgen receptor (Kelley, 1981). Because gonadal development is independent of thyroxine blockage and because exogenous androgen cannot overcome the blockade, it is likely that thyroxine regulates androgen sensitivity rather than androgen secretion itself.

Literature Cited

Gorlick, D., and Kelley, D. (1987). J. Comp. Neurol. 254, 614-627.

He, W.-W., Fischer, L., Sun, S., Bilhartz, D., Zhu, X., Young, C., Kelley, D., and Tindall, D. (1990). Biochem. Biophys. Res. Comm. 171, 697-704.

Kelley, D. (1981) J. Comp. Neurol. 199, 221-231.

Kelley, D. B, and Dennison, J. (1990). J. Neurobiol. 21, 869-882.

Kelley, D., Sassoon, D., Segil, N., and Scudder, M. (1989). Developmental Biology 131, 111-118.

Leloup, J., and Buscaglia, M. (1977). CR Acad. Sci. 284, 2261-2263.

Marin, M., Tobias, M. and Kelley, D. (1990). Development 110, 703-71.

Nieuwkoop, P. and Faber, J. (1956). "Normal table of Xenopus laevis (Daudin)". North-Holland, Amsterdam.

Robertson, J., Watson, J. and Kelley, D. (1991). Soc. Neurosci. Abstr. 17, 1320.

Sassoon, D. and Kelley, D. (1986). Am. J. Anat. 177, 457-472.

Sassoon D., Gary, G. and Kelley, D. (1987). J. Neurosci. 7, 3198-3206.

Sassoon, D., Segil, N. and Kelley, D. (1986). Dev. Biol. 113, 135-140.

Schonenberger, N. and Escher, G. (1988). Dev. Br. Res. 40, 253-260.

Simpson, H. Blair, Tobias, Martha L. and Kelley, Darcy B. (1986). 244, 430-444.

Tobias, M. L., Marin, M., and Kelley, D. B. (1991a). Developmental Biology 147, 251-259.

Tobias, M. Marin, M. and Kelley, D. (1991b) Developmental Biology 147, 260-270.

Sexual Differentiation of Brain and Behavior in Birds (Arthur P. Arnold and Barney Schlinger)

In mammals, a great deal of research suggests that all sex differences in behavioral and neural phenotype result from the differential secretion of androgens by the gonads. Testosterone or its metabolites (including estradiol formed in brain) cause masculine differentiation, and the lack of androgens allows feminine differentiation. The voluminous mammalian studies have strongly influenced similar research in birds.

Current evidence suggests two conflicting theories of the process of avian sexual differentiation of brain and behavior. One line of evidence, predominantly from studies on quail, ducks, and chickens, suggests that sexual differentiation results from differential secretion of estrogen. The embryonic ovary is thought to secrete more estrogen than the embryonic testes, and this estrogen acts to facilitate feminine development and suppress masculine development. This concept is the reverse of mammalian sexual differentiation, in that an ovarian hormone actively feminizes instead of a testicular hormone actively masculinizing.

A different pattern has been found in differentiation of the neural song system in zebra finches. Males of this species sing a courtship song that females lack, and brain regions controlling song are much larger in males than in females. Treatment of females with estrogen after hatching causes them to develop a more masculine neural song system, and to sing as adults. This result suggests a pattern of differentiation similar to mammals, in that the male brain is exposed to higher levels of estrogen during development, and that estrogen normally masculinizes the brain. However, various attempts to test this concept of sexual differentiation have been equivocal: (a) several antiestrogens fail to block masculine development when given to young male zebra finches; (b) there is some disagreement in the literature concerning the presence of sex differences in plasma estrogens early in post-hatching life, in that two labs do not find any difference but one lab finds that males have higher levels of estrogens than do females; (c) current (incomplete) evidence suggests that brain regions controlling song do not possess high levels of estrogen receptors.

To complicate matters, estrogen treatment of young male zebra finches can cause demasculinization of copulatory behaviors, in a manner similar to the quail pattern of sexual differentiation. Thus, it is possible that in the same species estrogens have both masculinizing and demasculinizing actions,

perhaps by acting at different times of development, or by acting simultaneously on different brain regions.

In adult male zebra finches, castration does not reduce the rather high circulating levels of estrogen. Aromatase (estrogen synthetase) is not found in testes or adrenals, but is extremely active in brain, especially the telencephalon. The brain takes up androgen from blood, converts it to estrogen, and releases estrogen into blood. Thus, we conclude that the brain is the major source of estrogen synthesis in male zebra finches. This finding suggests that the male masculinizes itself during development. Paradoxically, males and female thus far appear to have similar levels of aromatase in brain, and there is no current proof for the hypothesis that the male neural song system is exposed to higher levels of estrogen or androgen than the female song system. Further work is needed for an adequate testing of this hypothesis.

The importance of estrogen in sexual differentiation of zebra finches, together with the cerebral origin of estrogen in this species, suggests that pattern of neural sexual differentiation in birds (and hence in other vertebrate groups) may not always conform to the mammalian pattern as we currently understand it.

Use of the Brazilian Gray Short-Tailed Opossum in Studies on Neuronal Development and Sexual Differentiation of the Brain
(Carol D. Jacobson)

Our laboratory uses the Brazilian gray short tailed opossum (Monodelphis domestica) which was developed as a laboratory animal by John Vandeberg at the Southwest Foundation for Research and Education. Adult animals weigh about 100 grams. Immature pups are born after a two-week gestational period at which time they crawl to the mother's nipples (she is pouchless) and remain attached to a nipple for about two weeks. Development of the fetal-like animals proceeds, while being extremely accessible to study. We have determined that differentiation of the gonads takes place following the time of birth; alterations of the gonads follows topical application of estradiol benzoate (EB) during the early postnatal period; neurogenesis continues well into the postnatal period; and sex differences exist similar to what has been shown for the classical laboratory rat.

Our laboratory is presently interested in neuropeptide systems and steroid receptor systems in the adult and developing brazilian opossum. One of the main techniques we use is immunohistochemistry. The three main systems under study are cholecystokinin (CCK) containing pathways, galanin (GAL) containing pathways and studies on estrogen receptor (ER) regulation.

Cholecystokinin: We have studied the anatomical localization of CCK-like immunoreactivity (CCK-IR) in somata and fibers in the medial preoptic area (MPA) and anterior hypothalamus. Similar to what has been shown in the laboratory rat, we have observed that there is a sexually dimorphic distribution of CCK immunoreactive (male > female) elements within the MPA of this small marsupial.

In ontogeny studies, we have determined that the earliest expression of CCK-IR was found in fibers in the dorsal brainstem at 5 days of postnatal age (pn). Cell bodies were found to contain CCK as early as day 10 pn. A broad spectrum of patterns of onset of CCK expression was observed in the opossum brain. Interestingly, the sexually dimorphic distribution appears as soon as CCK is present in the MPA which occurs between day 25 and 35 pn.

Estrogen Receptor Expression: In relation to understanding the sexual differentiation of the CCK immunoreactive circuit we have begun a series of immunohistochemical studies using a monoclonal antibody generated against the estrogen receptor (Abbott H222). In these studies we have obtained data indicating that the levels of estrogen receptor like immunoreactivity (ER-IR) are affected by the presence or absence of the gonads. Further studies have shown that prolonged exposure of female opossums to EB decreases ER-IR when compared to animals in estrus or following gonadectomy. In addition we have found that all areas of the brain are not equally responsive to regulation by estrogen treatment.

In ontogeny studies, we have obtained data indicating that the number of cells that contain ER-IR increases in the brain through day 60 pn in all regions that will contain ER-IR in the adult opossum. Additionally, the first appearance of estrogen receptors in the brain occurs in the hypothalamus at 10 days pn. Most regions that contained ER in the adult will contain ER immunoreactivity by day 15 pn. Thus, ER are present before the sex difference in CCK like immunoreactivity appears.

Galanin: We have examined the distribution of galanin like immunoreactivity (GAL-IR) in the brain of the adult and developing opossum brain. In these studies, we have observed that there are nuclear groups which contain GAL-IR in cell bodies and fibers throughout the adult brain. In the developing brain, GAL-IR was seen as early as 1 day pn in the developing hypothalamus and brainstem. By days 5 and 10 pn, there was a robust expression of GAL-IR in specific regions of the brain. Since neurogenesis and brain morphogenesis are actively occurring postnatally in the opossum, galanin may be playing a role in the differentiation of specific regions of the brain, some of which will have sexually dimorphic functions in adulthood.

In conclusion, we believe that using the Brazilian opossum significant advances can be made in understanding the processes of vertebrate development and sexual differentiation.

Sex Differences in Vasopressin Systems (Geert J. De Vries)

During restricted periods in development, gonadal hormone levels, notably those of testosterone, determine whether mammals will be more likely to display masculine or feminine behaviors. This sexual differentiation appears to be reflected in the development of striking sex differences, for example, in the size of particular nuclei, in patterns of dendrites, and in the distribution of synapses in the brain. Such sex differences do not only invite one to relate structure to function, but since one can manipulate these mechanisms by changing gonadal hormone levels, they also offer prime opportunities to unravel mechanisms of brain development. The complexity of the sexually dimorphic areas, however, often makes it difficult to understand how these areas contribute to sex differences in brain function and by which cellular and molecular processes they have become differentiated.

One way of dealing with this complexity is to focus on specific neurotransmitter systems in sexually dimorphic areas. This might indicate, for example, which systems in particular contribute to the sex differences, and how these systems hook up with other areas in the brain. For example, the BST and MA are involved in sexually dimorphic functions such as the regulation of gonadotropin release and male sexual behavior. In both areas there are distinct dimorphisms involving the size, cell number, synapse distribution and dendritic branching. A number of neurotransmitter/neuropeptide systems may contribute to these differences. This abstract will focus on the vasopressin (AVP) neurons of the BST and MA.

Males show more AVP neurons than females and, likewise, the projections of these neurons to areas such as the lateral septum and midbrain central grey are much denser in males than in females. Steroid hormones dramatically influence the vasopressin-immunoreactive projections of the bed nucleus of the stria terminalis (BST) and the medial amygdaloid nucleus (MA). In both sexes, gonadectomy eliminates AVP mRNA labeling and AVP immunostaining of BST and MA neurons. In adult males, it takes a week before vasopressin mRNA levels drop to almost zero, but it takes over two months before all vasopressin immunoreactivity has disappeared from the terminals.

If males and females are given similar levels of testosterone in adulthood, they still show the same sex differences as untreated males and females. Neonatal manipulations of gonadal hormone levels indicated that around postnatal day 7 higher levels of testosterone in males induce

this sex difference. A number of differences seem to underlie the sex difference in vasopressin staining in the BST and MA. In addition to differences in the absolute number of cells that can make vasopressin, these cells also appear to differ in their sensitivity to testosterone metabolites. Dihydrotestosterone--which by itself does not restore AVP mRNA labeling in gonadectomized rats--increases AVP mRNA labeling in males that were also treated with estradiol, while it has no effect on the AVP mRNA labeling in females that were also treated with estradiol.

The vasopressin projections of the BST and MA may be involved in such functions as aggression and sexual behavior. A number of observations support this notion, for example, the nature of the hormonal effects on the AVP projections, lesion and stimulation studies involving the source of these projections, and psychopharmacological studies involving intracerebral injections of AVP and its analogues. It is highly unlikely that the actions of the steroid-sensitive AVP neurons would be confined to these functions. These same pathways are also influenced by osmotic challenges and changes in body temperature as well as during pregnancy in females. This suggest that vasopressin pathways can orchestrate a set of changes in a number of hormone-sensitive functions, for example, during seasonal changes or during pregnancy, both of which induce changes in gonadal hormone levels. In fact, vasopressin levels in these pathways decrease in photoperiodic animals when daylength shorten. In addition, in several rodent species, vasopressin levels are increased in BST and MA projections of pregnant maternal females, which may, for example, be tied to the osmotic challenges that these animals face.

Sex steroid effects on, and sex difference in, vasopressin pathways appear to be a phylogenetically primitive principle. Many other mammals and even non-mammalian vertebrates show sex differences or sex steroid effects in homologous pathways. For example, vasotocin pathways in amphibian, reptile and bird brains are sexually dimorphic and influenced by sex steroids in adulthood. In amphibians, central vasotocin levels also show seasonal fluctuations. The finding that, in amphibians, central vasotocin regulates the clasping response, which is a component of male sexual behavior, suggests that regulation of reproductive functions may have been preserved as function of steroid-sensitive vasopressin pathways in higher vertebrates.

Sexually Dimorphic Behavioral Control of LH and LHRH Secretion in the European Ferret (Michael J. Baum)

In numerous non-primate mammalian species the ability of estradiol to elicit a pre-ovulatory LH surge has been shown to be sexually dimorphic. Likewise, the ability of sex steroids tonically to inhibit LH secretion is sexually dimorphic, with

females being more sensitive than males to the feedback action of steroids. Over the past several years my co-workers and I have studied sex differences in the control of LH secretion in the ferret, a carnivore in which the female's preovulatory LH surge normally occurs in response to coital stimulation as opposed to a steroidal signal. In our initial studies Carroll, Erskine, and I found that the receipt of intromissive stimulation is required for a preovulatory LH surge in estrous female ferrets; receipt of neck grips and mounts without actual penile insertion fails to augment LH secretion. By contrast, achieving intromission fails to influence LH secretion in breeding male ferrets. This sex difference in the ability of intromissive stimulation to elicit an LH surge persisted in gonadectomized ferrets which were treated chronically with a low dosage of estradiol. Pituitary responsiveness to exogenous LHRH was similar in male and female ferrets, suggesting that a sex difference in the ability of genital, somatosensory input to stimulate forebrain LHRH neurons likely accounts for the differential LH response to mating. Support for this latter view comes from the results of two recent studies by Lambert, Rubin, and myself. In one experiment, the release of LHRH from the mediobasal hypothalamus of breeding ferrets was monitored *in vitro* following receipt (females) or achievement (males) of an intromission. In females killed 0.25h after onset of an intromission, the basal release of LHRH was significantly reduced in comparison with unmated female controls. No such difference in LHRH output was noted in mated versus unmated males killed after the same interval. This implies that in females the genital stimulation derived from intromission causes a rapid, massive release of LHRH from nerve terminals in the median eminence. As a consequence, LHRH stores are depleted by the time that the *in vitro* release of peptide was monitored beginning 0.25h after intromission onset. In a second study expression of the immediate-early gene, *c-fos*, was indirectly monitored by using immunocytochemical methods to visualize its nuclear protein product, FOS, in double-labelled, LHRH-immunoreactive neurons. Increased neuronal expression of *c-fos* has been shown to be a useful marker of neuronal activation in polysynaptic circuits following a variety of naturally occurring, sensory stimuli. In our study receipt of intromissive stimulation by breeding female ferrets caused a significant increment in the proportion of LHRH-IR forebrain neurons which were co-labelled with nuclear FOS-IR, compared with unpaired control females. By contrast, an equivalent, low percentage of LHRH neurons was co-labelled with FOS-IR in mated and unpaired males. Significantly more FOS-IR neurons (not co-labelled with LHRH) were detected in the bed nucleus of the stria terminalis, the medial preoptic area, the doral-medial hypothalamus, and the medial amygdala of mated versus unpaired females. In males, mating augmented FOS-IR only in the medial amygdala. These results suggest that the sexually dimorphic pattern of LH secretion which occurs in ferrets after mating reflects a selective activation of LHRH neurons in the female forebrain.

This sex-specific increase in the responsiveness of LHRH neurons to mating may depend on input from a limbic circuit, operative only in females, which includes the medial amygdala, bed nucleus of the stria terminalis, and the medial preoptic area.

Roles for Post-Translational Mechanisms During Sexual Differentiation of Mammalian Brain (Stuart A. Tobet)

We are studying sexual differentiation of the brain from perspectives ranging from biochemical to morphological. Recently we have focused on asking how cells reach their destinations during development and determining what role(s) gonadal steroid signals play in interpreting their environment. Important roles may be played by post-translational mechanisms in the developing neuroendocrine brain.

Following their birth in olfactory placode, luteinizing hormone-releasing hormone (LHRH) containing neurons migrate across the developing cribriform plate and form a dispersed population in the basal forebrain in all vertebrates that have been examined. They utilize axons from vomeronasal and medial olfactory neurons as guides upon which to travel into the CNS. In rats, prior to the detection of LHRH containing neurons, beginning on E13, CC2-immunoreactive (CC2ir) glycoconjugates were observed on vomeronasal cells and axons and also on a dorsomedial subset of olfactory neurons and axons. As early as E13-14 CC2ir fibers extended into the rostral forebrain. On E15, CC2 antigen(s) were detected on a subset of LHRH immunoreactive (LHRHir) cell bodies in the rostral forebrain. In addition, LHRHir neurons were seen in close apposition to CC2ir fibers in both the nasal cavity and rostral forebrain. At E15-16 approximately 20% of the LHRHir neuronal population in the forebrain had CC2 epitope(s) on surfaces of cell bodies and growth cones. This percentage fell as the number of LHRHir neurons in the forebrain increased. These studies raise the possibility that CC2ir glycoconjugates provide a specific chemical guide for a subset of LHRH neurons along their migratory pathways (Schwarting et al., 1991; Tobet et al., submitted). If CC2ir glycoconjugates participate in LHRH neuronal migration through recognition by selective lectins then molecules that interfere with such carbohydrate-lectin interactions could alter migratory capacity. Thus, competitive inhibition may be provided by the appropriate oligosaccharides.

Other studies indicate the identity of a different glycoconjugate recognized by the AB-2 monoclonal antibody that could mediate migration in a medial lateral dimension along radial glial guides (Tobet and Fox, 1989). Antibody AB-2 recognizes several polypeptides which are distributed selectively in subcellular fractions from neonatal brain and also several acidic glycolipids. The affinity of the AB-2 immunoreactive (AB-2ir) polypeptides for the lectin wheat-germ agglutinin, and AB-

2ir with proteins, polar lipids, and sulfatide suggests that the epitope is a carbohydrate present in multiple cellular compartments. Testosterone treatment selectively decrease the detectable level of only the 195kD AB-2ir polypeptide. The potential significance of this result is underscored by the possibility that the 195kD AB-2ir polypeptide acts through radial glia to play a role in determining the sexually dimorphic residency of neurons in the preoptic area/hypothalamus (Tobet et al., 1991).

Other mechanisms for regulating cell position related to sexual differentiation in the CNS may involve selective signal transduction mechanisms. In this regard, we have examined the expression of the serine/threonine kinase Raf-1, the protein product of the proto-oncogene c-*raf*-1. Biochemical and immunocytochemical analyses have localized Raf-1 in embryonic rat brain regions and demonstrated hormonally-induced changes in Raf-1 expression (Whorf and Tobet, 1992). While much evidence suggests that carbohydrate expression can be species selective, phosphorylation mechanisms are more highly conserved. Thus, for the purpose of selectively targeting lamprey, carbohydrate approaches would be more promising.

Literature Cited

Tobet SA & Fox TO (1989) Sex- and hormone-dependent antigen immunoreactivity in developing rat brain. Proc Natl Acad Sci, USA 86:382-386.

Tobet SA, Whorf RC, Schwarting GA, Fischer I & Fox TO (1991) Differential hormonal modulation of brain antigens recognized by the AB-2 monoclonal antibody. Dev Brain Res 62:91-98.

Schwarting GA, Drinkwater DL, Crandall JE & Tobet SA (1991) Luteinizing hormone-releasing hormone (LHRH) neurons migrate along vomeronasal and olfactory axons which express CC2 glycoconjugates in rats. Soc Neurosci Abstr 17:427.

Whorf RC & Tobet SA (1992) Expression of the RAf-1 Protein in Rat Brain During Development and its Hormonal Regulation in Hypothalamus. J. Neurobiology, 23:In Press.

Tobet SA, Crandall JE & Schwarting GA (submitted) Relationship of migrating luteinizing hormone-releasing hormone (LHRH) neurons to unique olfactory system glycoconjugates in embryonic rats.

RESEARCH NEEDS IN SEA LAMPREY CONTROL

After the workshop participants made their presentations, a general discussion was held on what types of research could be done to improve our knowledge of sea lamprey biology and the methods of controlling them. The discussion focused primarily on sterilization methods, sex reversal techniques, and metamorphosis but also included discussions of other research needs. Specific

areas of research were listed and prioritized (high, medium, low). Contact persons (C.P.) were listed for specific areas of research for those who might want more detailed information in those areas.

I. Sterilization Methods (Any sterilization method must not affect spawning behavior or mating competitiveness. Basic research in reproductive biology of the sea lampreys is of the HIGHEST priority and may lead to additional effective sterilization methods).

A. Spawning-run adults

1. Bisazir--For safety reasons, there is a need to determine the minimum dose required to sterilize males. (HIGH priority; C.P.-Seelye, Hanson).

2. GNRH antagonist--Method would provide a cost effective (less than 3 cents per lamprey) and efficient method (one injection of GNRH in a polymer). GNRH antagonist would be non-toxic to humans and males could be injected where they are captured and released immediately after injection. (HIGH priority; C.P.-Sower).

3. Genetic Modification--It may be possible to create females that will produce sterile males. Might be able to produce transgenic fish by introducing deleterious genes into a genetic line. For example, you might destroy or change the acrosomal proteins in the sperm so the egg would not be fertilized. You may be able to not only get males that are not able to produce acrosomal proteins but you may be able to produce females whose male offspring are unable to produce these proteins. Information on what has been done to produce trangenic fish needs to be identified (library search) and a workshop on this method might be useful. (MODERATE priority; C. P.-Kelley, Anne Kapuscinski, Tom Chen).

4. Hybrid Crosses--In some animals there are a number of crossing types which produce sterile males among their progeny. These hybrids are likely to be fully competitive and may show increased competitive mating ability because of hybrid vigor. In lampreys however, if the hybrids are non-parasitic the size diferential between sea lampreys and the sterile hybrid would likely prevent them from spawning together. It probably would also be unsuitable to release sterile hybrid males if they were parasitic on fish and if the

hybrid females were fertile. (LOW priority; C.P.-Youson).

B. Embryos, prolarvae, or young of the year larvae.--If sea lampreys could be effectively sterilized in the laboratory during the embryo, prolarval, or early larval stage it would be possible to produce millions of sterile animals (see abstract by Gorbman). They could be mass produced and released into a "nursery" stream. The stream could be treated with a larvicide prior to the introduction of the sterilized larvae and an effective barrier would prevent spawning-run lampreys from entering the nursery area and repopulating it with unsterilized larvae. This appears to be the most promising method of obtaining suitable numbers of sterile animals once the natural population has been reduced to a very low number (Hanson and Manion 1980). Potential methods of sterilization include the following:

1. Hormones--Includes sex steroids, anti-steroids, steroid synthesis inhibitors, peptides, etc. These materials may or may not affect spawning behavior and mating competitiveness of the treated lampreys. Studies will have to be conducted to determine this. (MODERATE priority; C. P.-Gorbman, Docker, Sower).

2. Target germ cells--(LOW priority; C. P.-Kelley).

3. Triploidy--Induced triploidy offers a good opportunity to apply a simple method (with demonstrated success in a number of fish species) to create sterile sea lampreys. The method involves the induction of triploidy in eggs just after fertilization which results in individuals that are almost always sterile (probably due to the inability of germ cells to mature). In salmonids, only triploid males develop secondary sex characteristics during the spawning season. If the same is true for sea lampreys (which can be established through research), then such individuals may still compete with fertile males for females, thus providing a means to disrupt reproduction and control population size. The Oregon Cooperative Fishery Research Unit at Oregon State University has engaged in research on sex differentiation in fishes for several decades and have used ploidy manipulation techniques for almost 10 years. The technique to induce triploidy consists of applying a thermal, pressure, or chemical shock to the egg shortly

after fertilization. The shock causes retention of the second polar body and individuals develop with a third set of chromosomes. Successful induction of triploidy can be indirectly assessed in blood samples from immature animals by examination of the size of erythrocyte nuclei. (MODERATE priority; C. P.-Fitzpatrick,Carl Schreck)

4. Bisazir Immersion--Spawning-run sea lampreys can be sterilized by immersion in aqueous solutions of bisazir (Hanson, 1981). It may be possible to sterilize embryos, prolarvae, or larval lampreys too. (MODERATE priority; C. P.-Seelye, Hanson).

5. Selective carbohydrates--alter GNRH neuron migration. (MODERATE priority; C. P.-Tobet).

6. $KC10_4$--(MODERATE priority; C. P.-Youson).

7. Density Factors--If sterile larval lampreys are placed in streams with naturally occurring larval populations, the sex ratios of both sterile (both sexes sterilized) and untreated larval populations may shift to predominately male populations which would be beneficial to a sterile male program. (MODERATE priority; C. P.-Docker).

II. Sex Reversal (Combined With Sterilization) If a monosex culture of all male larvae could be produced, the number of sterile larvae that would have to be produced and released in a sterile-male program could be greatly reduced since sterile females are of no value. The subsequent predation on fish would also be reduced.

A. Basic Research--Need more information on sex recognition and need to determine which sex is the heterogametic sex. (HIGH priority; C. P.-Fitzpatrick, Docker, Ed Donaldson).

B. Steroids--(HIGH priority; C. P.-Fitzpatrick, Docker, Gorbman, Ed Donaldson).

C. Androgenesis--(HIGH priority; C. P.-Fitzpatrick).

D. Steroid Synthesis Inhibitors--(HIGH priority; C. P.-Arnold).

E. Triploidy--The general method used to induce triploidy, called chromosome set manipulation, can be modified to produce all-female (gynogenesis) or all-male

(androgenesis) diploid populations. (MODERATE priority; C. P.-Fitzpatrick, Carl Schreck).

F. Environmental Factors--Studies by Docker and Beamish have proposed that sex determination in the least brook lamprey (Lampetra aepyptera) is density dependent. There is a need to investigate factors that determine sex in larval sea lamprey populations. It may be possible to use the observed changes in sex ratios as an indicator of the effectiveness of the control program. Also, if decreased density decreases the percentage of males in the natural population this will eventually increase the efficiency of the sterile-male release program by increasing the ratio of sterile to normal males. (MODERATE priority; C. P.-Docker, Youson, F. W. H. Beamish).

G. Differential Mortality--If an all male culture of sea lamprey larvae can be produced by finding a method to kill only females, this would be useful as part of a program that release sterile larvae into nursery streams. The use of satiety peptides (CCK) was suggested. (LOW priority; C. P.-Jacobson).

III. Metamorphosis

A. Basic Knowledge--There is a need to investigate and determine the factors that affect transformation. For example, we may then be better able to evaluate the potential contribution of a specific larval population to adult stocks. It appears that these factors are highly selective since only a portion of the larvae of sufficient size and age will metamorphose in a given year. (HIGH priority; C. P.-Youson).

B. Environmental Influences--The effects of environmental influences (temperature, density, etc.) on transformation must be determined before the possibility of controlling metamorphosis using these factors can be evaluated. It may also be useful to know the minimum temperature required for transformation in order to better evaluate the potential of lake populations (particularly in the cold waters of Lake Superior) of larvae to transform. (HIGH priority; C. P.-Youson, Docker).

C. Methods to Prevent Metamorphosis or Induce Early Metamorphosis--The effects of hormones, hypophysectomy, heat shock proteins, temperature, condition factors, sex, etc. on transformation is necessary so we will better understand the transformation process. New and

novel methods of lamprey control may result from these studies. (HIGH priority; C. P.-Youson, Jean Joss).

IV. Alternate Model System--Because of the long life cycle of the sea lamprey, it was suggested that it would be useful to find and use a species of lampreys with a shorter life cycle (1-2 years) for preliminary studies. The species chosen should also be easy to culture in the laboratory and be non-parasitic. If such a species is not available it would be useful to develop better culture methods for sea lampreys in the laboratory and develop ways to produce 2-3 year old transformers at a suitable rate. It would also be useful to select and not treat a stream in which sea lampreys are known to transform in about 3 years and use that as a source for experimental animals. It was also suggested that cell culture techniques might be developed which would be suitable for certain experiments. (HIGH priority; C. P.-Kelley, Youson, Docker, Seelye, Hanson).

V. Other Studies

A. Reproductive Behavior--Sex pheromones need to be identified. Steroids, prostaglandins, gonadal products (analyze steroids from ripe gonads), and peptides should be examined as possible pheromones. Studies should be conducted on other possible attractants (bile, iron, etc.), repellents (spit, etc.), and fright reactions in lampreys. (HIGH priority; C. P.-Gorbman, Youson).

B. Feeding Behavior--May be useful to determine role and localization of peptides in lampreys. May be able to produce nonparasitic sea lampreys. (LOW priority; C. P.-Jacobson).

CONCLUSIONS

It became apparent during the workshop that even though the sea lamprey has been studied intensively over the years, there is still much to be done. The amount of research that could be done in the fields of sterilization techniques, sex determination, and metamorphosis alone is quite impressive. In order to facilitate research of this type, the following suggestions were made:

1. Because of the long life cycle of the sea lamprey there is a need for either an alternate test species with a shorter life cycle or improved methods of culturing sea lampreys in the laboratory.

2. Because of the long life cycle, there is a need for a long-term commitment by the Great Lakes Fishery Commission for studying basic lamprey biology and

conducting research. Many studies cannot be completed in one year.

3. If we are going to come up with a clever biological way of controlling sea lampreys, we are going to have to understand lamprey biology. For example, more information is needed on lamprey reproductive biology and metamorphosis. The work being done by Gorbman, Youson, Docker, and Sower should be given high priority and other scientists should be encouraged to submit research proposals in collaboration with one of these investigators.

4. Although the specific areas of research discussed at the workshop were given a priority rating (high, medium, or low) the Commission and its subgroups must ultimately decide what research should be pursued. If more information is needed on certain studies, it may be useful to contact the person or persons suggested in this workshop report. Several researchers expressed a desire to work on lampreys and would provide additional information or prepare research proposals if asked to do so.

Literature Cited

Hanson, L. H. 1981. Sterilization of sea lampreys (Petromyzon marinus) by immersion in an aqueous solution of bisazir. Can. J. Fish. Aquat. Sci. 38:1285-1289.

Hanson, L. H., and P. J. Manion. 1980. Sterility method of pest control and its potential role in an integrated sea lamprey (Petromyzon marinus) control program. Can. J. Fish. Aquat. Sci. 37:2108-2117.