Molecular approaches to study the genetic regulation of the fish reproductive system

MARTIN P. SCHREIBMAN[1]
Department of Biology, Brooklyn College, City University of New York, 2900 Bedford Avenue, Brooklyn, NY 11210, U.S.A.

MANFRED SCHARTL
Theodor-Boveri Institut für Biowissenschaften, University of Würzburg, Germany

KLAUS D. KALLMAN
Department of Ichthyology, American Museum of Natural History, New York, U.S.A.

AND

LUCIA MAGLIULO-CEPRIANO
Department of Biology, Brooklyn College, City University of New York, 2900 Bedford Avenue, Brooklyn, NY 11210, U.S.A.

Introduction

The traditional approaches to the study of biology (e.g., anatomy, physiology, embryology, genetics) have given way to new tactics and disciplines that reflect the progress and sophistication of methods of investigation generated by advanced technology. A new generation of scientists has emerged who use a scientific jargon that is strange to the ears of veteran investigators. Modern methodology and narrowing our fields of vision have led to isolation and compartmentalization of methods of biological study. It is our perception that in recent years too many of us in the scientific community have been working in circles of ever decreasing diameter so that we have become experts in, and restricted our efforts to, specific technologies and highly esoteric fields of investigation—some of us have even chosen to devote our lives to specific molecules. It is our premise that as a result, microcosms of study have resulted, each having their sphere of technique and savvy, as Fig. 1 suggests. We believe that too many of us have lost sight of the "big picture" and the fundamental question. However, the isolated subdisciplines would, if joined together, yield more rapid major advances in comprehending broad scientific issues and problems. We need to refocus on questions of significance and determine what the information derived from our experimental manipulations means in the context of a whole, living organism. In this regard, fundamental knowledge of the genetics and physiology of an appropriate test organism is an essential prerequisite for designing and comprehending molecular biological protocols.

The genus *Xiphophorus*, a group of freshwater teleosts, has been touted for many years as an extraordinary research model for addressing fundamental questions covering several biological and medical disciplines (Powers 1989; Schreibman et al. 1986, 1987, 1991). In this treatise, *Xiphophorus* will be used to illustrate the value of combining the apparently disparate fields of genetics, physiology, and molecular biology (the primary interests of the authors of this article) to comprehend the nature of the reproductive system. Thus, the major objectives of this paper are (*i*) to introduce (or reintroduce) an animal with special research utility; (*ii*) to define genetic and neuroendocrine control of reproduction as a problem of fundamental importance and significance; (*iii*) to review the current state of our knowledge in three spheres of study, genetics, neuroendocrine physiology, and molecular biology; and (*iv*) to present new ways of looking at old problems, essentially suggesting melding separate spheres of influence into a single one with a specific, defined focus.

The genus Xiphophorus

The genus *Xiphophorus*, family Poeciliidae, comprises 22 species, several of which have commanded special scientific interest. The two species that have been studied most extensively for 80 years are *Xiphophorus maculatus*, the southern platyfish, and *Xiphophorus helleri*, the green swordtail.

Xiphophorus maculatus occurs in the lowlands of southern Mexico, from the Rio Jamapa, Veracruz, across northern Guatemala into Belize (Kallman 1975). Three sex chromosomes, X, W, and Y, occur in natural populations of *X. maculatus*; thus females may be WX, WY, and XX and males XY and YY (Table 1; Kallman 1975, 1984, 1989). Unlike mammals, the sex chromosomes of *Xiphophorus* are similar to each other and possess homologous loci (Kallman 1975; Nanda et al. 1992, 1993). Several stocks have been inbred for more than 80 generations, thus providing us with fish with a high degree of homozygosity. Platyfish in natural populations range in standard length (SL) from 18 to 45 mm (approximate mean, 23 mm) and in body weight between 0.5 and 3 g, for both sexes.

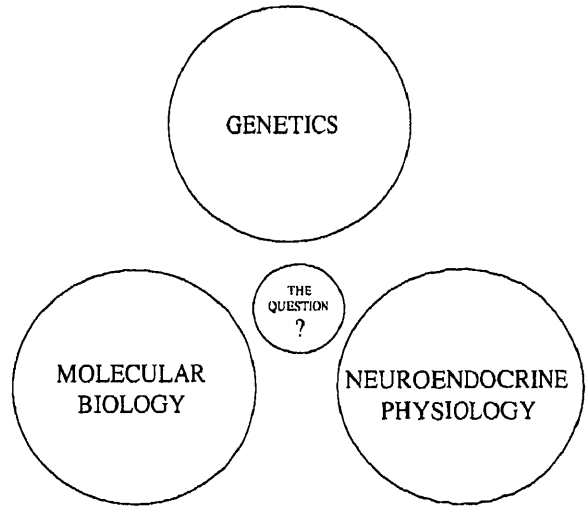

FIG. 1. The question (see text).

[1]Author to whom all correspondence should be addressed.

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TABLE 1. Sex-determining mechanisms and *P* gene polymorphism in 10 species of *Xiphophorus*

Taxon	Females	Males	*P* gene polymorphism
X. maculatus	WY WX XX	XY YY	On X and Y, W has early factor
X. milleri	XX	XY	On X and Y
X. variatus	XX	XY	Restricted to Y
X. andersi	XX	XY	Restricted to Y
X. pygmaeus	XX	XY	Restricted to Y
X. nigrensis	XX	XY	Restricted to Y
X. cf. *montezumae*	XX	XY	Restricted to Y
X. cortezi	XX	XY	Restricted to Y
X. alvarezi	WY	YY	Factors on W and Y not the same
X. helleri	Not known	Not known	Adult size of males is correlated with sex ratio and *P* gene polymorphism

NOTE: From Kallman (1989).

TABLE 2. Advantages of *Xiphophorus* as an experimental model

- Studied for more than 60 years in the laboratory and field. Well in excess of 1000 papers published
- The only vertebrate known in which a key life-history trait (age at sexual maturity and perhaps longevity may be controlled by natural variations at a single locus
- The *Xiphophorus* gene map is one of the most extensive among vertebrates in number of assigned genes, ranking close to the maps of man, rat, mouse, and cow
- Most extensive longitudinal study (from birth to death) of the interaction of brain, pituitary, and gonadal factors related to reproductive physiology. Reproductive system structure and function similar in many ways to that of mammals
- Extensive study of the molecular biology of the sex chromosomes, especially of the locus regulating normal and abnormal pigment formation
- The only system in which melanomas can be produced spontaneously by simple genetic crosses of two species of *Xiphophorus*
- Serves as a laboratory model for applied studies in aquaculture, gravitational biology, controlled life-support systems, and analysis of ecological perturbations
- The genetics laboratory (formally at New York Aquarium) maintains 40 stocks of 20 species of *Xiphophorus*. Each stock is well characterized genetically, especially for pigmentation, biochemical markers, sex determination, life-history traits, geographical distribution, and histocompatibility
- Some stocks highly inbred (brother–sister matings for 80 generations); some have become homozygous and do not reject tissue transplants exchanged among members of the same stock
- The *Xiphophorus* genome is thought to represent the basic vertebrate genome (Ohno 1967), making it attractive for evolutionary studies

TABLE 3. Gene-regulated endocrine-based processes in *Xiphophorus*

- Age and size at puberty – BPG axis development
- Sex determination
- Growth rate and final body size
- Number and quality of gametes
- Secondary sex characters
- Longevity
- Pigment patterns
- Endocrine abnormalities (e.g., melanomas, thyroid tumors, pituitary cysts)

TABLE 4. The nine known *P* alleles of *X. maculatus*, their associated pigment factors, and X or Y linkage and geographical origin

P factor	Pigment factor, sex chromosome, and origin
$*P^1$	Sp^1-X Jp, *DrSd*-X Jp, Sp^9-Y Cp, N^2-Y Cp
$*P^2$	+-Y Bp (2*x*), *ArSr*-Y Jp
P^7	*Dr*-Y Bp, +-Y Bp
$*P^3$	Sp^8-Y BP (2x), *Mr*-Y Bp (2x), *MrSd*-Y Bp, *Rs*-Y Bp, *Av*-Y Bp, *Ir*-Y Bp, +-Y Bp, +-X Up
P^9	+-Y Bp, *f-Sd*-X Up
P^8	N^1-Y Bp, *TySr*-Y Bp, *CpoSd*-Y Bp, *Ir*-Y Bp
P^6	*Asr*-Y Cp, +-Y Bp
$*P^4$	*Br*-Y BP
$*P^5$	N^1-X Bp

NOTE: The *P* factors are listed in order of size at sexual maturation. The pigment genes have been described previously (Kallman 1975). Geographical origin: Jp, Río Jamapa; Cp, Río Coatzacoalcos; Up, Río Usumacinta system; Bp, Belize River. From Kallman (1989).

**P* factors previously identified.

The green swordtail has a somewhat wider distribution. It is found from the Rio Nautla, Veracruz, eastward towards Guatemala, Belize, and into Honduras. It inhabits small streams from near the coast into the highlands at elevations approaching 1400 m above sea level. Green swordtails are broadly sympatric with platyfish in the lowlands, but hybrids have never been found. Mature males range in size from 24 to 65 mm (SL) and their mass varies correspondingly. At maturity, males develop a long "sword" from elongated ventral caudal fin rays, an appendage that is missing in the platyfish.

Xiphophorus serves to combine the disciplines of genetics, neuroendocrine physiology, and molecular biology. Our choice of *Xiphophorus* as a model system is appropriate because this fish is the only vertebrate known in which a key life-history trait, that is, age at sexual maturation, and apparently life-span, may be controlled by natural variations at a single locus. Table 2 provides additional reasons for our bias towards *Xiphophorus*.

Genetics

In Table 3 we present examples of associations between genome and physiological processes in *Xiphophorus*.

In 1973 we reported that differences in the age and size at sexual maturation (puberty) are determined by a multiple allelic series at a gene locus on the sex chromosome; we termed this the *P* locus (Kallman and Schreibman 1973; Kallman et al. 1973). Each of the alleles is closely linked to pigment genes, which thus serve as phenotypic markers for *P*.

Nine *P* alleles have now been identified in *X. maculatus*

TABLE 5. Age at sexual maturity at five homozygous genotypes of platyfish

P genotype	Pigment pattern	Sex	*n*	Maturity (weeks, ±SE)	Treatment[a]
P^1P^1	$DrSp^9$	♂♂	23	7.9±0.2	*i*
P^1P^1	$DrSp^1$	♀♀	8[b]	8.0	*m*
P^2P^2	*SrSr*	♂♂	8	13.5±0.2	*i*
P^2P^2	*SrSr*	♂♂	4	10.8±0.8	*i*
P^2P^2	*SrSr*	♂♂	12	14.6±0.3	*m*
P^3P^3	*IrIr*	♂♂	17	12.6±0.3	*m*
P^4P^4	*BrBr*	♂♂	46	26.5±0.6	*m*
P^4P^4	*BrBr*	♂♂	3	24.7±2.67	*i*
P^5P^5	N^1N^1	♀♀	164[c]	34–104	*i*, *m*

NOTE: Modified from Kallman and Borkoski (1978).
[a] *i*, fish raised in isolation; *m*, mass culture.
[b] Fish are 23 mm in length.
[c] Some females still with undeveloped gonads at 73 weeks.

(Table 4; Kallman 1989), four in the swordtail *X. multilineatus*, and two or three alleles in several other species of the genus. As far as is known, each *P* allele is species specific. Table 5 lists examples of various *P* gene combinations, the pigment genes that mark them, and their effect on the age and size at sexual maturation. There is no evidence for preferential association of certain *P* factors with certain color genes; each *P* allele can be linked to a variety of pigment factors. For example, P^1 is limited to four color genes and P^3 to seven (Kallman 1989). Basic Mendelian genetic crosses enable us to "synthesize" offspring with a variety of combinations of *P* alleles linked to different pigment genes. In addition, because of the special sex-determining mechanisms in *Xiphophorus*, we are also able to generate various gender ratios, including all-male progeny.

It is not entirely correct to state that the *P* locus controls the age at maturity or the size at maturity, because these two demographic parameters are intimately linked. In *X. maculatus*, if individuals of a given genotype are raised under different environmental conditions, they will mature at widely different ages and sizes. This is, of course, the well-known phenomenon of environment–genotype interaction. However, two different *P* genotypes raised under identical conditions will always mature at different ages and sizes. However, in other species (e.g., *X. multilineatus*) the *P* genotypes are more clearly correlated with size at maturity rather than with age. By varying rearing conditions the age at maturity can be changed, but size at maturity stays constant. In other words, by changing the conditions one modulates the age (the growth rate) at which the genetically specified size at maturity is attained.

Because it is awkward in discussions to talk about *P* alleles that control age and size at maturity, as well as for brevity's sake, we will maintain in this presentation that *P* factors control age at maturity and we will refer to the alleles as early and late *P* factors.

Regardless of the underlying genetic relationship between size and age at maturation, in males the growth rate declines sharply or ceases altogether at sexual maturation; therefore individuals with genotypes for "early" maturation remain relatively small throughout their adult life-span, whereas those with "late" genotypes will grow relatively large (Kallman and Schreibman 1973). The size difference between genotypes is less well marked in females, who continue to grow after maturation.

Two patterns of inheritance can be recognized in the genus. In some species (e.g., *X. maculatus* and *X. milleri*) alleles for both early and late maturation may be present on both X and Y chromosomes. Individuals can be bred that are either homozygous or heterozygous for early or late *P* factors, and early and late genotypes are not restricted to a particular sex. The *P* locus is most closely linked to the macromelanophore gene and also to genes for xanthophore and pterinophore pigmentation and the sex locus.

The genetic cross presented below illustrates how the *P* gene system works. This cross is used frequently in our protocols, for it provides early- and late-maturing siblings of both sexes. This enables us to separate chronological age from physiological age in our studies of reproductive system development. A female of *X. maculatus* heterozygous for an early (P^1) and a late (P^5) factor was mated to a male heterozygous for two early factors (P^1 and P^2). The following macromelanophore factors served as marker genes for the *P* alleles: *Sp* (spot sided) for P^1, *N* (nigra) for P^5, and *Sr* (stripe sided) for P^2. The cross can then be written as follows.

$$P_1\text{: X-}N\text{X-}Sp\ (P^5P^1) \times \text{X-}Sp\text{ Y-}Sr\ (P^1P^2)$$

F_1 (The progeny derived are males and females that mature at different sizes and ages.):

Males

X-*Sp* Y-*Sr* (P^1P^2)

X-*N* Y-*Sr* (P^5P^2)

Females

X-*Sp* X-*Sp* (P^1P^1)

X-*N* X-*Sp* (P^5P^1)

For example, males with P^1P^2 genotype mature at 14 ± 0.3 weeks and 21.4 ± 0.3 mm and males with P^5P^2 at 33.2 ± 0.8 weeks and 35.2 ± 0.5 mm. A similar relationship between age and size exists in early-maturing (P^1P^2) and late maturing (P^5P^1) females (Kallman and Schreibman 1973; Kallman and Borkoski 1978).

A different pattern of inheritance is present in many species of swordtails, in which the *P* gene polymorphism is restricted to the Y chromosome. However, in *X. helleri* the pattern of sex determination is still unknown (see Table 1). The X chromosomes of these species always carry the early *P* alleles, whereas the Y chromosomes carry the factors for early, intermediate, or late maturation. Consequently, some males mature at the

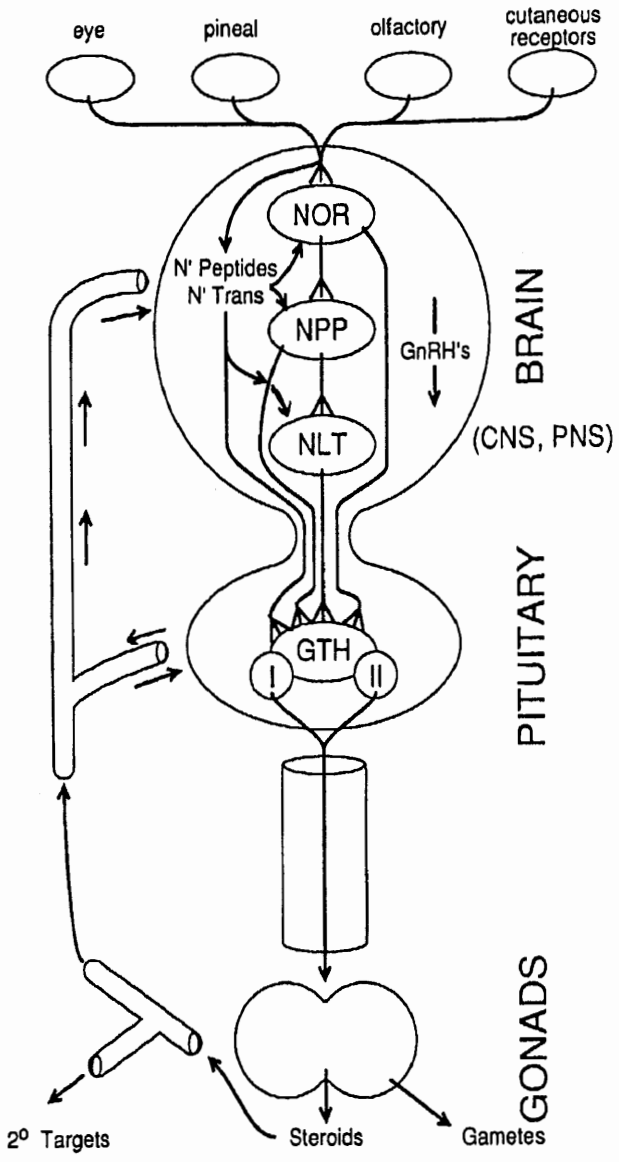

FIG. 2. A schematic representation of the BPG axis depicting its relationship with receptor organs of environmental cues. Hormones involved in this axis are indicated within their appropriate organ; arrows indicate paths of movement of humoral agents and feedback controls (see text). CNS, central nervous system; PNS, peripheral nervous system; 2°, secondary; N' Trans, neurotransmitters; N' peptides, neuropeptides.

same age and size as females, whereas other males may take twice as long to attain maturity, and size varies correspondingly. Because the polymorphism is restricted to the Y chromosomes, age and size at maturity of males is inherited paternally. Individuals homozygous for late maturation do not occur under natural conditions and also cannot be bred in the laboratory. In the absence of crossing-over, factors that effect behavior and pigmentation have become linked to specific *P* alleles.

Neuroendocrine physiology

We set out to understand the neuroendocrine basis of the genetic control of sexual maturation and, hopefully, to clarify the nature of the *P* gene. Table 6 lists the specific features of the reproductive system of *Xiphophorus* that makes it attractive for study. Our work on the genetic and neuroendocrine

TABLE 6. *Xiphophorus maculatus*: features of the reproductive system

Internal insemination by copulation
Internal fertilization
Sperm storage within female
Follicular gestation
Multiple broods from a single insemination
Free-swimming newborn
Usually 20–40 animals per brood
28 days between broods on year-round basis
Age at maturity genetically determined (2–12 months) at *P* locus marked by linkage to body pigment genes
Sexually dimorphic body structure
Gonopodium development, an index of androgen production
Gametogenesis until death
Average life-span of 2.5 years

NOTE: From Schreibman et al. 1990.

TABLE 7. Neuroendocrine–physiological time table for *Xiphophorus* development

Available for:
multiple forms of GnRH
GTH I, GTH II
neuroregulatory peptides (complete for at least 7)
steroids
gametogenesis
secondary sex characters
maturational changes in receptor organs

control of reproductive system development in *Xiphophorus* has been summarized and the significance assessed in recent reviews (Halpern-Sebold et al. 1986; Schreibman et al. 1986, 1987, 1990, 1991; Schreibman and Margolis-Nunno 1987). Our research has established the sequence of interactive developmental events (a "calendar," so to speak) in the brain–pituitary–gonad (BPG) axis that culminates in the ability of the organism to produce viable gametes (see Table 7).

The neuroendocrine agents responsible for regulating BPG axis structure and function (e.g., gonadotropin-releasing hormone (GnRH), gonadotropic hormones (GTHs), steroid-synthesizing enzymes) are already present in neonatal platyfish (Schreibman et al. 1982*a*, 1982*b*, 1991). What was needed was to clarify the specific role of the genome and identify the mechanisms that orchestrate the activities leading to sexual maturity.

We have found that there are distinct changes in the distribution and relative quantity of immunoreactive (ir) peptides, neurotransmitters, and pituitary and gonadal hormones during the life-span of platyfish. These and other physiological alterations can be related to genotype, age (physiological and (or) chronological), and sex and are especially dramatic at the time of reproductive system maturation (cf. Schreibman et al. 1982*a*, 1982*b*, 1982*c*, 1987, 1991; Halpern-Sebold and Schreibman 1983).

The relationship among three brain nuclei (nucleus olfactoretinalis, NOR; nucleus preopticus periventricularis, NPP; nucleus lateralis tuberis, NLT) containing immunoreactive gonadotropin-releasing hormones (ir-GnRHs), the pituitary gland, the gonad, and the sensory organs that convey environmental cues to the BPG axis is illustrated in Fig. 2. It has been demonstrated that numerous ir-fibers emanate in all directions from the NOR, and processes can be clearly delineated between this nucleus and the two other ir-GnRH-containing nuclei, the

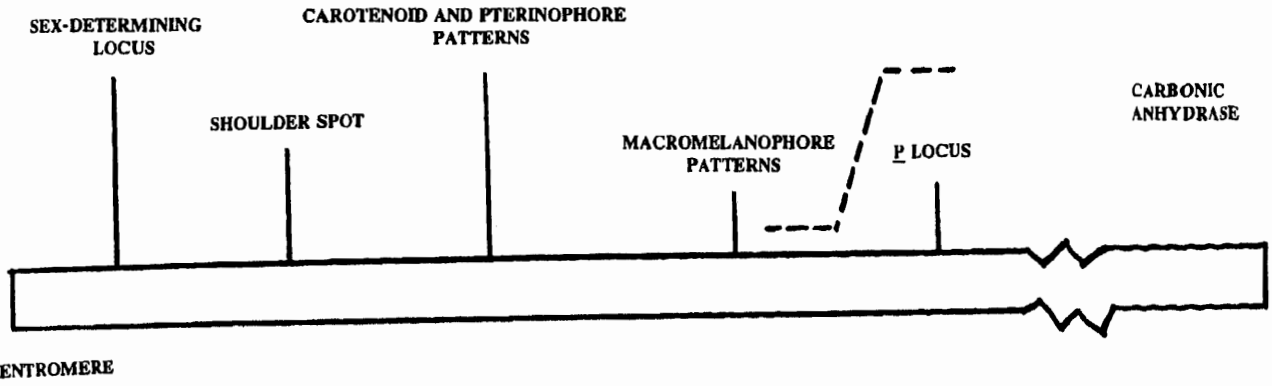

FIG. 3. A schematic representation of the *Xiphophorus* sex chromosome. Broken line between macromelanophore patterns (*Xmrk*) and *P* locus indicates unresolved positional relationship of two loci (see Fig. 4).

pineal gland (habenula nucleus region), the olfactory tract, the optic nerve, and the pituitary gland (Schreibman et al. 1982*a*; Halpern-Sebold and Schreibman 1983; Münz et al. 1981; Demski 1984). ir-GnRH appears in the NOR early in development in both early (at 5 weeks) and late (at 9 weeks) maturers. At the initiation of the maturation process, ir-GnRH appears in the NPP and very soon after in the NLT. These events directly precede the proliferation of the gonadotropes in the ventral caudal pars distalis (vCPD) of the pituitary gland and the subsequent maturation of the gonads. The attainment of sexual maturity in males is revealed by the final transformation of the anal fin into a gonopodium, a process dependent upon increasing levels of androgen.

The pituitary gland of neonatal platyfish is characterized by equal areas of neurohypophysial and adenohypophysial tissue. With the exception of the gonadotropes in the vCPD, all cell types characteristic of the mature gland are present in their characteristic regional distribution in neonatal fish (Schreibman 1964, 1986). Cells containing both ir-gonadotropin (ir-GTH; reactive with antibody generated against the β-subunit of a number of teleostean GTHs) and ir-GnRH (reactive with antibody generated against the synthetic mammalian peptide) can be identified in 1-week-old fish in the periodic acid − Schiff positive (PAS^+) cells of the pars intermedia (PI) and in clusters of cells in the lateral regions of the caudal pars distalis (CPD); gonadotrophs of the vCPD are represented by only a few scattered, isolated ir-cells (Schreibman et al. 1982*a*). The gonadotropes of the PI are numerous at 1 week of age, while those of the vCPD begin to appear just prior to the onset of sexual maturation and soon increase markedly in number, size, and activity.

There is a direct relationship between the number of ir-GnRH-containing neurons in the brain and the number of ir-GTH-containing CPD and PI cells in the pituitary in both early and late maturers from 1 week to adulthood. In adult early and late maturers, the number of ir-cells in the PI is similar, but late maturers have significantly fewer ir-cells in the CPD (Halpern-Sebold et al. 1986; Halpern-Sebold and Schreibman 1983). In both genotypes, the initial appearance of ir-GnRH in the NPP and NLT of the brain occurs just prior to the proliferation of gonadotropes in the CPD.

The results of our recent studies (Magliulo-Cepriano and Schreibman 1992; Magliulo-Cepriano et al. 1994) demonstrate that variant forms of GnRH (salmon (s), chicken II (cII)) and GTH (GTH βI, GTH βII) are present at defined stages of *Xiphophorus* development and that they are associated with specific morphological regions of the brain and pituitary gland. sGnRH and GTH βI are already clearly present by 2 weeks of age. cIIGnRH and GTH βII are not seen until the onset of puberty. Similarly, there is a specific regional and chronological distribution in *Xiphophorus* of neuropeptides and neurotransmitters that have been implicated in GnRH and GTH synthesis and release (Magliulo-Cepriano et al. 1993; Magliulo-Cepriano and Schreibman 1993; Margolis-Nunno et al. 1985, 1987; Schreibman and Halpern 1980). These studies suggest, therefore, that different forms of GnRH and GTH are associated with different aspects of reproductive system development and function and that neuropeptides are, in some yet to be determined way, related to coordinating BPG axis interaction.

In platyfish gonads, the enzymes essential for steroid synthesis (3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase) are seen in the Leydig cells of the youngest males examined (3 days old). At a similar age in females, these enzymes are localized in the stroma of the ovaries, which essentially comprise previtellogenic oocytes (Schreibman et al. 1982*b*; M.P. Schreibman and R. van den Hurk, unpublished). At the time of sexual maturation, in both males and females, cells containing these enzymes become more numerous and more intense in their histochemical response. Soon they display a pattern of enzyme distribution characteristic of fully developed gonads (Schreibman et al. 1982*b*; M.P. Schreibman and R. van den Hurk, unpublished).

Molecular biology

The fundamental question persists: What is the where, when, how, and why of the *P* gene? In recent years there has been a surge of activity resulting from the development of sophisticated technology enabling us to approach the questions that have been with us for so long. Figure 3 depicts the current working model of the structure of the sex chromosome of *Xiphophorus*. This information has essentially been derived from genetic linkage data and molecular mapping studies (see, for example, Anders et al. 1973; Kallman 1975; Morizot et al. 1991; Schartl and Adam 1992).

The long-term studies to understand the genetic regulation of melanoma formation in *Xiphophorus* have provided a giant step in isolating and characterizing the *P* gene. In many species of *Xiphophorus*, individuals exhibit melanophore patterns composed of large, intensely black pigment cells (macromelanophores) arranged as irregular spots superimposed on a uniform olive-gray body coloration formed by small black

pigment cells (micromelanophores) and xanthophores arranged in a reticular pattern (Gordon 1927). More than 60 years ago it was discovered that hybrids derived from matings between platyfish with macromelanophores on their dorsal fin and unspotted swordtails (*X. helleri*) spontaneously develop malignant melanomas (Gordon 1927; Hausler 1928; Kosswig 1928). Shortly thereafter, it was recognized that the occurrence of tumors in hybrids is due to a single locus (the macromelanophore locus) of *X. maculatus* that "interacted" with the *X. helleri* genome (Gordon 1931; Kosswig 1929). This interaction was subsequently defined as the effect of modifying genes (presence of intensifying genes contributed by *X. helleri* and (or) absence of repressing genes from *X. maculatus*) in the hybrid genome, which act specifically on the macromelanophore locus (Gordon 1958; Atz 1962; Kosswig 1965; Zander 1969; Kallman 1970). However, the situation is more complex. The same macromelanophore patterns occur in all the different platyfish populations. Yet the macromelanophore alleles are population specific, as are their modifier systems. Therefore, a more appropriate view is that macromelanophore factors and their regulatory units within a population constitute a co-adapted genetic system that brings about species-specific patterns (Kallman 1970).

In a typical cross, a female *X. maculatus* that carries the X-chromosomal macromelanophore gene *Sd* (spotted dorsal) is mated to *X. helleri*, which does not carry the corresponding gene. The F_1 hybrid shows enhancement of the Sd phenotype. Backcrossing of the F_1 hybrid to *X. helleri* results in offspring that segregate into 50% that have not inherited the *Sd* locus and are phenotypically like the *X. helleri* parental strain and 50% that carry the macromelanophore locus and develop melanoma. The severity of the atypical pigment cell hyperplasia ranges from benign (pigment cell overgrowths) in some individuals (phenotype like the F_1 hybrids) to highly malignant growths in others, where the melanomas are invasive, exophylic, and fatal to the individual. Based on the classical crossing experiments involving the *Sd* factor of the Jamapa stock of *X. maculatus*, a genetic model has been proposed to explain tumor formation in *Xiphophorus* (Ahuja and Anders 1976). The sex-linked macromelanophore locus was formally equated to a melanoma oncogene locus, whose critical constituent was designated "tumor-gene" (*Tu*). Melanoma formation then was attributed to the uncontrolled activity of *Tu*. In nontumorous fish, *Tu* activity was proposed to be negatively controlled by regulatory genes or tumor-suppressor genes (*R* genes, corresponding to the modifying genes mentioned above). For the crossing experiment outlined above, this means that *X. maculatus* contains the *Tu*–*Sd* locus on the X chromosome and the corresponding major *R* on an autosome, while *X. helleri* is proposed not to contain this particular *Tu* locus and its corresponding *R*. According to the model, backcrossing of the *Tu*-containing hybrids to *X. helleri* results, in effect, in the progressive replacement of *R*-bearing chromosomes from *X. maculatus* by *R*-free chromosomes of *X. helleri*. The stepwise elimination of regulatory genes is thought to allow expression of the *Tu* phenotype, leading to benign melanoma if one functional allele of *R* is still present or malignant melanoma if *R* is absent (for review see Anders et al. 1984). However, it should be noted that it is similarly compatible with the crossing data to attribute *Tu* activity to the presence of intensifying genes contributed by *X. helleri* chromosomes in the hybrid genome. Whether this model also applies to other macromelanophore factors and (or) to "foreign" species other than *X. maculatus* has been questioned (Veilkind et al. 1989). This suggests still other tumor-suppressor systems.

TABLE 8. Isolating and understanding the *P* gene

1. Establish a physical map of the *P*-gene region of the sex chromosome using rare cutting restriction enzymes and pulsed-field gel electrophoresis
2. Identify CpG islands within the physical map. These islands are indicative of structural genes
3. Clone CpG islands and flanking DNA
4. Identify candidate genes using Southern analysis of various *P* alleles
5. Confirm that the candidate gene is responsible for the P phenotype, using select genetic crosses and gene transfer along with evaluation of reproductive system development.

Reintroduction of the regulatory genes of *X. maculatus* by crossing melanoma-bearing hybrids to *X. maculatus* leads to a return to the normal phenotype in the progeny of the succeeding backcross generations, always using *X. maculatus* as the recurrent parent (Anders et al. 1984). This demonstrates that the macromelanophore gene (or the oncogene *Tu*) itself remains structurally unaltered during the process of activation via hybridization. Four years ago the gene at the *Tu* locus was cloned (Adam et al. 1988; Schartl 1988; Wittbrodt et al. 1989). The *Tu* gene is called *Xmrk*, the *Xiphophorus* melanoma receptor *k*inase. This was accomplished by applying "reverse genetics," determining the location of the chromosome, finding nearby genetic markers, and isolating the correct candidate genes.

How can this information on *Xmrk* and melanoma help in the isolation and characterization of the *P* gene? So far no unequivocal unique gene product for the *P* locus has been defined, either on the RNA or protein level. This precludes cloning of the *P* gene by classical methods of recombinant DNA technology. The single remaining alternative for a molecular approach is the application of positional cloning or, as termed earlier, reverse genetics. The first step for positional cloning is to localize precisely the gene or locus to a chromosomal region. The next step, once the chromosomal position of the gene in question has been completely resolved, would be the isolation of molecular marker sequences. Such molecular markers will provide the access to a physical map. In a third step this map can be used to sort and orient genomic clones that cover the region of interest by chromosome walking or chromosome jumping, using cosmid or YAC (yeast artificial chromosomes) clones. Thus, the whole chromosomal region can be isolated molecularly. Identification of transcripts encoded in that region will ultimately lead to the isolation of candidate genes (see Table 8).

For the positional cloning strategy the conditions in *X. maculatus* are very good, Mapping the *P* gene locus to the sex chromosomes in close proximity to a specific pigmentation locus has already been accomplished through our genetic studies (Kallman and Schreibman 1973; Kallman and Borkoski 1978). The whole complex resides towards the telomeric region. This has important implications for our cloning approach towards the *P* gene. What is encouraging is that in the chromosomal region that is close to the telomere, recombination frequencies are equal to much shorter physical map distances than on the rest of the chromosome or even in the region close to the centromere. One centiMorgan (cM) in *Xiphophorus* averages to approximately 600 kilobases (kb) (Morizot et al. 1991). This means that the *P* gene is much closer to the *Xmrk* gene, which is our starting point for the chromosome walk, than we had previously expected.

After the first segment of this strategy was set, our next goal was to find molecular marker sequences and to get a physical

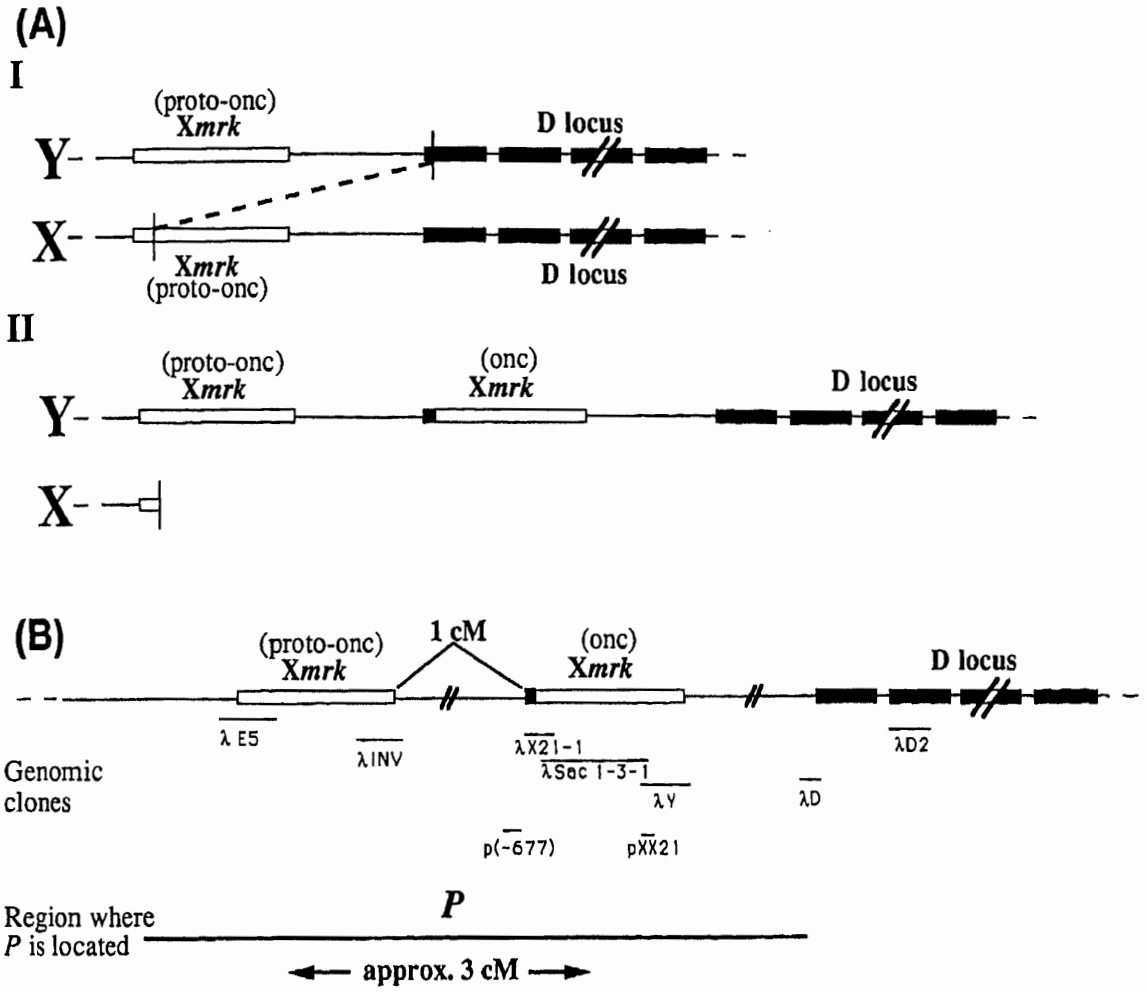

FIG. 4. (A) A model for the evolution of a duplicated locus near the *P* gene. I. Situation in ancestral *Xiphophorus*. *X* and *Y*, sex chromosomes or their precursors; broken line, site of meiotic nonhomologous recombination event. II. Result of the recombination, the mutant X is lost in meiosis; Y, recent sex chromosome of *Xiphophorus* (see text). proto-onc, proto-oncogene. onc, oncogene. (B) Schematic map of *X. maculatus* sex chromosome: loci, clones available, proposed *P* location (see text).

map of the sex chromosomal region around the *P* locus. First, information was provided through our studies on the melanoma-inducing oncogene, which is not only closely linked to the macromelanophore locus or even part of it but which is also in the vicinity of the *P* gene (see above). The melanoma oncogene was cloned by applying reverse genetics. A molecular marker (pXX21) was isolated (Adam et al. 1988). Using this marker as a probe, a full-length transcriptional clone was isolated from a melanoma cDNA library. More than 30 kb of the genomic region was then cloned. This spans the entire transcribed region and up to 1 kb of the 5′ upstream regulatory region (Adam et al. 1991). Large parts have been sequenced, thus providing the first sequence tagged site (STS) on the sex chromosome of *X. maculatus*

Southern blot analysis with molecular probes from the *Xmrk* oncogene as a probe on DNA from a large number of fish with and without macromelanophore patterns obtained from natural populations and laboratory stocks, as well as their hybrids, revealed the following genomic organization of this chromosomal region. The *Xmrk* gene exists in two copies in the genome of *Xiphophorus*. One copy is present on each of the sex chromosomes (including W, X, and Y of *X. maculatus*) as a bona fide proto-oncogene serving in a so far unknown physiological function. It encodes a membrane receptor tyrosine kinase. *Xiphophorus maculatus*, as well as several other species of *Xiphophorus* that carry a macromelanophore locus with a predisposition for melanoma formation, has a second copy of *Xmrk*. This copy is an oncogene and is responsible for the neoplastic transformation of cells of the macromelanophore lineage in hybrids (e.g., with *X. helleri*).

Linkage analysis revealed that both copies of *Xmrk* are located within a distance of approximately 1 cM on the sex chromosomes (Fig. 4; Schartl 1990). This distance is roughly equivalent to 600 kb of physical map distance. A complementary DNA (cDNA) and genomic clones for the proto-oncogene have been cloned and partially sequenced, thus providing the second STS (sequence tagged site) on the sex chromosome. It became now clear that the *Xmrk* oncogene arose through a gene duplication event during the evolutionary history of the genus. A nonhomologous recombination event occurred with another locus that we have designated *D* with exon 1 of proto-oncogene *Xmark*, exactly 3′ of codon 10. This event generated the additional copy of *Xmrk* with a new promoter (Fig. 4). Suppression of this new *Xmrk* promoter (which is in fact derived from the *D* locus) by the tumor suppressor locus *R* (located on linkage group V) in parental *X. maculatus* and its deregulation in hybrids explain the genetics of melanoma formation in *Xiphophorus* (Adam et al. 1993). The *D* locus is

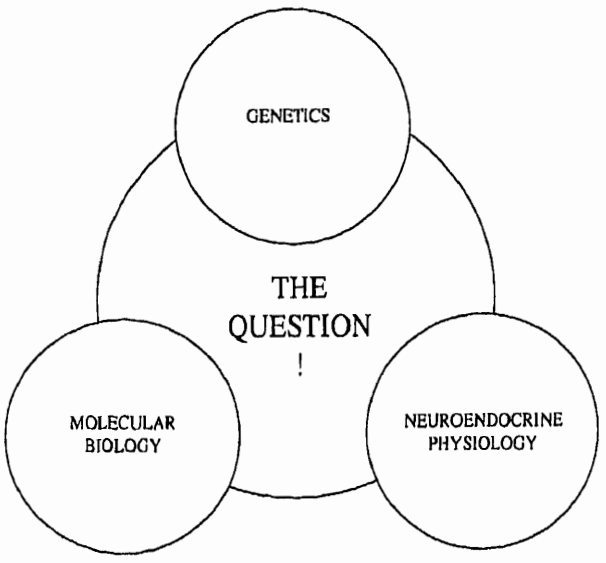

FIG. 5. THE QUESTION !!!! (see text).

under scrutiny in the laboratory. It is of potential importance, because it might provide the third STS on the sex chromosome. The gene duplication model would predict that the *D* locus is also linked to *Xmrk*. The *D* locus is an amplified sequence in the *Xiphophorus* genome. Studies using double fluorescence labelling of *Xmrk* and *D* locus probes are underway to show the linkage of both. Sequences from the *D* locus are already available. Genomic clones have been isolated and sequenced. Similarly, cDNA clones were obtained by screening a melanoma cell library. With the three molecular markers, *Xmrk* proto-oncogene, *Xmark* oncogene, and *D* locus, a region of the sex chromosome is covered to which within 1 cM at the borders the *P* gene is located as inferred from the combination of genetic linkage data and molecular mapping (Fig. 4). The next level of resolution that is needed for cloning the *P* gene is to establish a long-range map by pulsed-field electrophoresis. This has been started using rare cutting enzymes that produce fragments in the 100- to 500-kb range. The regions around the *Xmrk* proto-oncogene and oncogene have been mapped (Ziegler and M. Schartl, unpublished). Enzymes that produce larger fragments will be used to link both regions. In parallel, a genomic library in yeast artificial chromosomes with *X. maculatus* DNA as an insert has been produced (J. Wittbrodt and M. Schartl, unpublished). From this library the clones spanning the sex chromosomal region marked by the above-mentioned STS or *Xmrk* and eventually *D* can be isolated. These clones can then be used for the identification of transcripts and finally for finding candidate genes for the *P* locus. This information will be most useful in our attempt to isolate the *P* gene. We propose to use a similar approach, which is summarized in Table 8. Finally, it is absolutely essential to have a good animal system to ultimately test if an isolated DNA sequence has the characteristics of what we expect for the *P* locus.

Summary

In summary, we have an animal model that has enabled us to identify a specific locus on an identified chromosome that is directly correlated with a physiological process. We can produce combinations of *P* alleles in animals of either gender at will. The reproductive system has been dissected at various levels of the BPG axis and studied in order to create an neuroendocrine time table for development. In addition, we have utilized modern molecular biological technology and thinking to close in on the *P* locus. We have taken major steps towards resolving how to isolate and characterize the *P* gene; this is certain to advance our understanding of how genotype regulates physiological processes.

Finally, we have seen how three fields of investigation have been pursuing similar questions but within their own spheres of influence. It has been our intention to demonstrate that the energies, expertise, and technologies of these seemingly diverse fields of study need to be integrated within the sphere of the BIG QUESTION to be answered (Fig. 5). In this way we anticipate rapid advancement towards a comprehensive understanding of genomic control of reproduction and, indeed, of the physiological process in general!

Adam, D., Wittbrodt, J., Telling, A., and Schartl, M. 1988. RFLP for an EGFL-receptor related gene associated with the melanoma oncogene locus of *Xiphophorus maculatus*. Nucleic Acids Res. **16**: 7212.

Adam, D., Maueler, W., and Schartl, M. 1991. Transcriptional activation of the melanoma inducing *Xmrk* oncogene in *Xiphophorus*. Oncogene, **6**: 73–80.

Adam, D., Dimitrijevic, N., and Schartl, M. 1993. Tumor suppression in *Xiphophorus* by an accidentally acquired promoter. Science (Washington, D.C.), **259**: 816–819.

Ahuja, M.R., and Anders, F. 1976. A genetic concept of the origin of cancer, based in part upon studies of neoplasms in fishes. Prog. Exp. Tumor Res. **20**: 380–397.

Anders, A., Anders, F., and Klinke, K. 1973. Regulation of gene expression in the Gordon-Kosswig melanoma system I, II. *In* Genetics and mutagenesis of fish. *Edited by* J.H. Schroeder. Springer-Verlag, New York. pp. 33–63.

Anders, F., Schartl, M., Barnekow, A., and Anders, A. 1984. *Xiphophorus* as an in vivo model for studies on normal and defective control of oncogenes. Adv. Cancer Res. **42**: 191–275.

Atz, J.W. 1962. Effects of hybridization on pigmentation in fishes of the genus *Xiphophorus*. Zoologica (N.Y.), **47**: 153–181.

Demski, L.S. 1984. The evolution of neuroanatomical substrates of reproductive behavior: sex steroid and LHRH-specific pathways, including the terminal nerve. Am. Zool. **24**: 809–830.

Gordon, M. 1927. The genetics of a viviparous top-minnow *Platypoecilus*: the inheritance of two kinds of melanophores. Genetics, **12**: 253–283.

Gordon, M. 1931. Hereditary basis of melanosis in hybrid fishes. Am. J. Cancer, **15**: 1495–1519.

Gordon, M. 1958. A genetic concept for the origin of melanomas. Ann. N.Y. Acad. Sci. **71**: 1213–1222.

Halpern-Sebold, L.R., and Schreibman, M.P. 1983. Ontogeny of centers containing luteinizing hormone-releasing hormone in the brain of platyfish (*Xiphophorus maculatus*) as determined by immunocytochemistry. Cell Tissue Res. **229**: 75–84.

Halpern-Sebold, L., Schreibman, M.P., and Margolis-Nunno, H. 1986. Differences between early and late maturing genotypes of the platyfish (*Xiphophorus maculatus*) in the morphometry of their immunoreactive LHRH containing cells. A developmental study. J. Exp. Zool. **240**: 245–257.

Hausler, G. 1928. Über Melanombildungen bei Bastarden von *Xiphophorus maculatus* var. rubra. Klin. Wochenschr. **7**: 1561–1562.

Kallman, K.D. 1970. Different genetic basis of identical pigment patterns in two populations of platyfish, *Xiphophorus maculatus*. Copeia, **3**: 472–487.

Kallman, K.D. 1975. The platyfish *Xiphophorus maculatus*. *In* Handbook of genetics. *Edited by* R.C. King. Plenum Press, New York. pp. 81–132.

Kallman, K.D. 1984. A new look at sex determination in poeciliid

fishes. *In* Evolutionary genetics of fishes. *Edited by* B.J. Turner. Plenum Press, New York. pp. 95–171.

Kallman, K.D. 1989. Genetic control of size at maturity in *Xiphophorus*. *In* Ecology and evolution in livebearing fishes (Poeciliidae). Chap. 9. *Edited by* G.K. Meffee and F.F. Snelson. Prentice Hall, Englewood Cliffs, N.J. pp. 163–184.

Kallman, K.D., and Borkoski, V. 1978. A sex-linked gene controlling the onset of sexual maturation in female and male platyfish (*Xiphophorus maculatus*), fecundity in females and adult size in males. Genetics, **89**: 79–119.

Kallman, K.D., and Schreibman, M.P. 1973. A sex-linked gene controlling gonadotrope differentiation and its significance in determining the age of sexual maturation and the size of the platyfish, *Xiphophorus maculatus*. Gen. Comp. Endocrinol. **21**: 287–304.

Kallman, K.D., Schreibman, M.P., and Borkoski, V. 1973. Genetic control of gonadotrop differentiation in the platyfish, *Xiphophorus maculatus* (Poeciliidae). Science (Washington, D.C.), **58**: 678–680.

Kosswig, C. 1928. Über Kreuzungen zwischen den Teleostiern *Xiphophorus helleri* und *Platypoecilus maculatus*. A. Indukt. Abstamm. Vererbungsl. **47**: 150–158.

Kosswig, C. 1929. Das Gen in fremder Erbmasse. Zuechter, **1**: 152–157.

Kosswig, C. 1965. Genetische Grundlagen des Polymorphismus. Zool. Anz. **175**: 21–50.

Magliulo-Cepriano, L., and Schreibman, M.P. 1992. Variant forms of GnRH in the platyfish from birth to sexual maturity. Am. Zool. **32**: 17.

Magliulo-Cepriano, L., and Schreibman, M.P. 1993. The distribution of neuropeptide Y and dynorphin immunoreactivity in the brain and pituitary gland of the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. Cell Tissue Res. **271**: 87–92.

Magliulo-Cepriano, L., Schreibman, M.P., and Blüm, V. 1993. The distribution of immunoreactive FMRF-amide, neurotensin and galanin in the brain and pituitary gland of three species of *Xiphophorus* from birth to sexual maturity. Gen. Comp. Endocrinol. **92**: 269–280.

Magliulo-Cepriano, L., Schreibman, M.P., and Blüm, V. 1994. Distribution of variant forms of immunoreactive gonadotropin-releasing hormone and beta gonadotropin I and II in *Xiphophorus* from birth to sexual maturity. Gen. Comp. Endocrinol. In press.

Margolis-Nunno, H., Halpern-Sebold, L.R., and Schreibman, M.P. 1985. Immunocytochemical localization of serotonin in the brain and pituitary gland of the platyfish, *Xiphophorus maculatus*. Cell Tissue Res. **240**: 311–314.

Margolis-Nunno, H., Schreibman, M.P., and Halpern-Sebold, L. 1987. Sexually dimorphic age-related differences in the immunocytochemical distribution of somatostatin in the platyfish. Mech. Ageing Dev. **41**: 139–148.

Morizot, D.C., Slaugenhaupt, S.A., Kallman, K.D., and Chakravarti, A. 1991. Genetic linkage map of fishes of the genus *Xiphophorus* (Teleostei: Poeciliidae). Genetics, **127**: 399–410.

Münz, H., Stump, W.E., and Jennes, L. 1981. LHRH systems in the brain of the platyfish. Brain Res. **221**: 1–13.

Nanda, I., Schartl, M., Feichtinger, W., Epplen, J.T., and Schmid, M. 1992. Early stages of sex chromosome differentiation in fish as analyzed by simple repetitive DNA sequences. Chromosoma (Berlin), **101**: 301–310.

Nanda, I., Schartl, M., Epplen, J.T., and Schmid, M. 1993. The primitive sex chromosomes in poeciliid fishes harbor simple repetitive DNA sequences. J. Exp. Zool. **265**: 301–308.

Ohno, S. 1967. Sex chromosomes and sex linked genes. Springer-Verlag, Berlin, Heidelberg, New York.

Powers, D.A. 1989. Fish as model systems. Science (Washington, D.C.), **246**: 352–358.

Schartl, M. 1988. A sex chromosomal restriction-fragment-length marker linked to melanoma-determining *Tu* loci in *Xiphophorus*. Genetics, **119**: 679–685.

Schartl, M. 1990. Homology of melanoma-inducing loci in the genus *Xiphophorus*. Genetics, **126**: 1083–1091.

Schartl, M., and Adam, D. 1992. Molecular cloning, structural characterization, and analysis of transcription of the melanoma oncogene of *Xiphophorus*. Pigm. Cell Res. Suppl. **2**: 173–180.

Schreibman, M.P. 1964. Studies on the pituitary gland of *Xiphophorus maculatus* (the platyfish). Zoologica (N.Y.), **49**: 217–243.

Schreibman, M.P. 1986. Pituitary gland. *In* Vertebrate endocrinology: fundamentals and biomedical implications. Vol. 1. *Edited by* P.K.T. Pang and M.P. Schreibman. Academic Press, New York. pp. 11–15.

Schreibman, M., and Halpern, L.R. 1980. The distribution of neurophysin and arginine vasotocin by immunocytochemical methods in the brain and pituitary gland of the platyfish, *Xiphophorus maculatus*. Gen. Comp. Endocrinol. **40**: 1–7.

Schreibman, M.P., and Margolis-Nunno, H. 1987. Reproductive biology of the terminal nerve (nucleus olfactoretinalis) and other LHRH pathways in teleost fish. *In* The terminal nerve (nervus terminalis) structure, function and evolution. *Edited by* L.S. Demski and M. Schwanzel-Fukuda. Ann. N.Y. Acad. Sci. **519**: 60–68.

Schreibman, M.P., Margolis-Kazan, H., and Halpern-Sebold, L.R. 1982*a*. Immunoreactive gonadotropin and luteinizing hormone releasing hormone in the pituitary gland of neonatal fish. Gen. Comp. Endocrinol. **47**: 385–391.

Schreibman, M.P., Berkowitz, E.J., and van den Hurk, R. 1982*b*. The histology and histochemistry of the platyfish (*Xiphophorus maculatus*) testis and ovary from birth to sexual maturity. Cell Tissue Res. **224**: 81–88.

Schreibman, M.P., Margolis-Kazan, H., Halpern-Sebold, L., and Goos, H.J.Th. 1982*c*. The functional significance of the nucleus olfactoretinalis in the platyfish, *Xiphophorus maculatus*. *In* Proceedings of the International Symposium on Reproductive Physiology of Fishes. *Edited by* C.J. Richter and H.J.Th. Goos. Pudoc, Wageningen, the Netherlands. p. 59.

Schreibman, M.P., Margolis-Nunno, H., and Halpern-Sebold, L. 1986. The structural and functional relationships between olfactory and reproductive systems from birth to old age in fish. *In* Chemical signals in vertebrates. Vol. 4. *Edited by* D. Duvall, D. Muller-Schwartze, and R.M. Silverstein. Plenum Press, New York. pp. 155–172.

Schreibman, M.P., Margolis-Nunno, H., and Halpern-Sebold, L. 1987. Aging of the neuroendocrine system. *In* Hormones and reproduction in fishes, amphibians and reptiles. *Edited by* D.O. Norris and J.E. Jones. Plenum Press, New York. pp. 563–584.

Schreibman, M.P., Holtzman, S., and Cepriano, L. 1990. The life cycle of the brain–pituitary–gonad axis in teleosts. *In* Progress in comparative endocrinology. Wiley-Liss Inc., New York. pp. 399–408.

Schreibman, M.P., Holtzman, S., and Margolis-Nunno, H. 1991. Maturation and aging of the reproductive system. *In* Vertebrate endocrinology: fundamentals and biomedical implications. Vol. 4A. *Edited by* P.K.T. Pang and M.P. Schreibman. Academic Press, New York. pp. 343–378.

Veilkind, J.R., Kallman, K.D., and Morizot, D.C. 1989. Genetics of melanomas in *Xiphophorus* fish. J. Aquat. Anim. Health, **1**: 69–77.

Wittbrodt, J., Adam, D., Malitschek, B., Maueler, W., Raulf, F., Telling, A., Robertson, S.M., and Schartl, M. 1989. Novel putative receptor tyrosine kinase encoded by the melanoma-inducing *Tu* locus in *Xiphophorus*. Nature (London), **341**: 415–421.

Zander, C.D. 1969. Über die Entstehung und Veränderung von Farbmustern in der Gattung *Xiphophorus* (Pisces). Mitt. Hamb. Zool. Mus. Inst. **66**: 241–271.