the University of Kansas to study the genera *Fundulus* and *Notropis*. Our thanks to both institutions. Our thanks also to Frank B. Cross and Sharon L. Dewey, University of Kansas, for their valuable comments on our earlier draft. Lorraine Hammer of the University Electron Microscope Facility kindly assisted us in the examination of materials presented here.

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A CELL CULTURE TECHNIQUE FOR CHROMOSOME PREPARATION IN CYPRINID FISHES.

—Chromosome studies in fishes have not been as successful as in other vertebrates, primarily because of the inability to consistently produce high quality metaphase spreads (Gold, 1979). Commonly used methods of direct chromosome preparation from solid tissues (McPhail and Jones, 1966; Gold, 1974; Kligerman and Bloom, 1977) can provide good metaphases from fishes with low numbers of relatively large chromosomes (Vervoort, 1980; Almeida Toledo et al., 1981; Kligerman, 1982). Most fishes, however, possess relatively large numbers of small chromosomes (Kligerman and Bloom, 1977; Gold, 1979) and the direct preparation methods often yield little information other than diploid number and rough estimates of chromosome arm number. Cell culture for chromosome studies offers several advantages: 1) large numbers of metaphases can be obtained; 2) chromosome morphology is generally better than obtained from direct tissue preparation; 3) several slides can be prepared from small individuals; and 4) specimens maintained in laboratory aquaria for long periods may still be used. These advantages will ultimately make cell culture a useful tool for improving the current status of fish chromosome research.

Success in obtaining metaphase preparations from lymphocyte, short-term monolayer (fibroblast and epithelial), and long-term cell culture has been reported for several fish species (Gold, 1979). Lymphocyte culture is preferred over short-term monolayer culture for obtaining chromosomes in terms of time and cost, but realistically can be used only when specimens are large enough to yield sufficient quantities of whole blood. Long-term cell cultures frequently become chromosomally heteroploid and are not recommended for general karyotyping (Regan et al., 1968; Chen and Ebeling, 1975). Herein, we describe a simple and reliable short-term monolayer cell culture technique for preparing chromosomes from small specimens of North American cyprinid fishes.

Materials and methods.—Fish were collected by seine from the Little Brazos River (Brazos County, Texas) and maintained in the laboratory in well aerated aquaria. To initiate primary cultures, the caudal fin and a few scales (10–15 for a 2 g specimen) are removed from each fish, rinsed briefly (10–15 sec) in 70% ethanol, and placed in separate 25 cm^2, 30 ml tissue culture flasks (Corning), viz., the scales are placed in one flask and the caudal fin is placed in another. Two ml of growth medium—Medium 199 (KC Biological) adjusted to pH 7.4 and fortified 20% with fetal calf serum (KC Biological) or 10% fetal calf serum and 10% newborn calf serum (KC Biological)—are added to the flasks. Flasks are then incubated at 30 C and monitored daily for explant outgrowth and contamination (bacterial or fungal) using a tissue culture (inverted) microscope. The pH of the growth medium is also monitored daily (via pH indicator dye) and kept between 7.2 and 7.6 by replacement of one-half of the volume with fresh growth medium. Antibiotics and antimycotics are never used in the culture procedure.

After 2–4 wks, when monolayer growth reaches confluency (bottom of culture flask completely covered with cells), the cells are subcultured. This entails removing the growth medium from the flask, washing the cells for 10 min in ca. 10 ml Hanks' base (Wolf and Quimby, 1969), detaching one-half of the cells from the

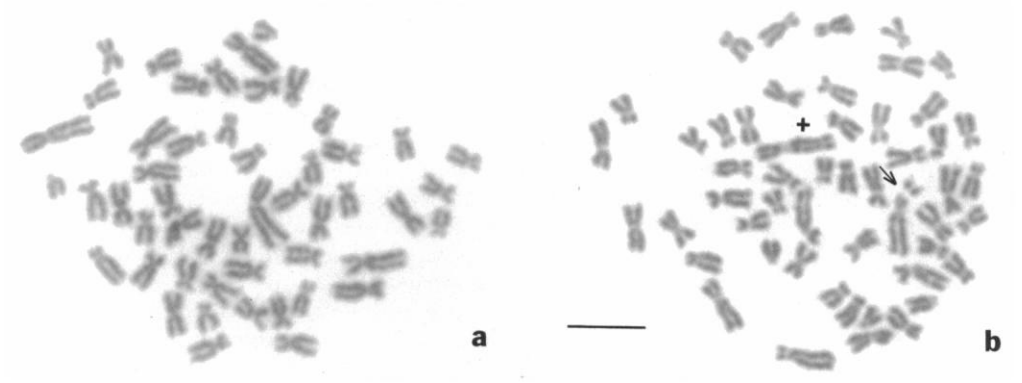

Fig. 1. Giemsa stained metaphase chromosomes from (a) *Notropis venustus*, and (b) *Notropis lutrensis*. Chromosomes were obtained from fin- and scale-initiated cultures, respectively. The *N. lutrensis* spread shows a heteromorphism (arrow) in the nucleolus organizer region or NOR (see Gold, 1984). The normal homologue is indicated by +. Bar equals 5 microns.

bottom of the flask with ca. 1 ml of 0.25% (v/v) trypsin (KC Biological) in Hanks' base, and transferring the detached cells to a 75 cm^2, 200 ml tissue culture flask (Corning) to which 10 ml of growth medium are added. Subculture flasks are incubated at 30 C, and monitored daily for cell attachment, growth, pH changes, and contamination. When cell growth reaches two-thirds confluency in a subculture flask (usually within two days), the flask is karyotyped.

One to four hours prior to harvesting subcultured cells for karyotyping, 0.01 ml of a 10 mg/ml stock colcemid (Gibco) solution (in Hanks' base) is added to the flask to arrest mitotic activity. The growth medium is then removed and the cells are washed 10 min in ca. 10 ml of Hanks' base. One-third to one-half of the cells are then detached from the bottom of the flask with 2–3 ml of 0.25% (v/v) trypsin in Hanks' base, and the detached cells transferred to a 15 ml conical centrifuge tube. The subculture flask containing the remaining (attached) cells is either discarded or incubated at 30 C with fresh growth medium (to be karyotyped again in a few days). The harvested cells are centrifuged at 40 × *g* for 2 min, the supernatant removed, and the cell pellet resuspended in 5 ml of 0.075 M KCl. Hypotonic incubation is for 30 min at 30 C, after which the suspension is centrifuged at 40 × *g* for 2 min. The supernatant is removed and the cells fixed for 5 min in methanol/acetic acid (3:1). The fixed cells are then centrifuged at 40 × *g* for 2 min and the fixative replaced at least once or twice prior to preparing slides. Slides are made by dropping suspended cells from a height of 1 cm onto cold, wet microslides, or by the slide warmer technique of Kligerman and Bloom (1977). Air dried preparations are stained in 5% Giemsa (in 10^{-2} M phosphate buffer, pH 6.8) for 5 min, rinsed briefly in distilled water, and air dried. All preparations are made permanent by clearing slides in xylene for 10 min followed by mounting in Permount.

Results and discussion.—Representative metaphases obtained from our technique are shown in Fig. 1. To date, four cyprinid species—*Notropis lutrensis, Notropis venustus, Pimephales vigilax,* and *Notemigonus crysoleucas*—have been karyotyped by the cell culture method described above, and high quality metaphase spreads (>100 per slide) showing good chromosome morphology (Fig. 2) were reliably obtained. At least 10 slides could routinely be prepared from a harvested subculture flask. The 2n = 50 chromosome number observed in greater than 90% of the well spread metaphases from harvested subcultures of the above four species is in agreement with Gold et al. (1980). Although we did not observe any quantitative or qualitative differences between metaphases from flasks in which the cells were harvested once and those in which the cells were harvested a second time, it should be noted that in vitro changes in chromosome morphology and ploidy levels have been documented in various long-term fish cell culture lines (Regan et al., 1968; Chen and Ebeling, 1975). Hence, we do not recommend the use of flasks in which cells have

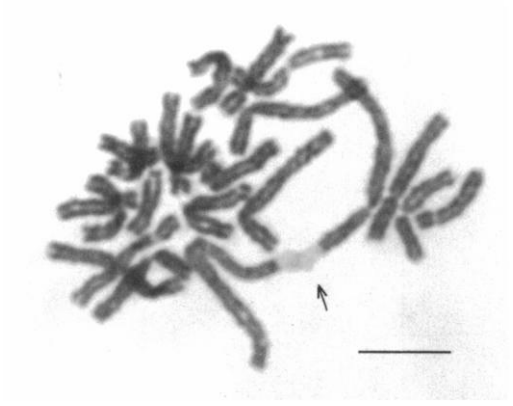

Fig. 2. Pro-metaphase chromosomes from *N. lutrensis* showing an association (arrow) of the NOR chromosomal material. Bar equals 5 microns.

been harvested (or subcultured) four or more times for studies where species specific karyotypes are desired.

In addition to fins and scales, we also tested kidney, spleen, heart and trunk musculature for culture potential. Manually-generated tissue fragments (Ojima, 1978) and trypsinized tissue suspensions (Ojima, 1978; Noga, 1979) were tested as well as intact tissues. Intact scale and caudal fin tissues were selected over all others (intact or otherwise) as they repeatedly yielded the most consistent monolayer cell growth. Success rates for obtaining chromosomes from cultures initiated from scales and caudal fins were ca. 50% and 70%, respectively.

Initiation of the primary cultures was the most critical part of the procedure; success here usually led to successful karyotyping. Factors we found limiting to cell growth and attainment of monolayer confluency in primary culture flasks were: 1) use of antibiotics or antimycotics in the growth medium; 2) bacterial or fungal contamination; 3) incubation temperatures that were too high or too low; 4) pH of medium too acidic or too basic; 5) use of excessive growth medium resulting in floating tissues and no outgrowth from the explants; and 6) large numbers of epithelial cells (epithelial cells sometimes died or stopped growing and were generally more difficult to culture than fibroblasts). Factors 1–5 were easy to control by never using antibiotics or antimycotics in growth media, by always practicing aseptic measures (flame sterilization of instruments, laminar flow hood, etc.), and by incubating flasks within optimal temperature and pH ranges of the fishes and using minimal amounts of growth medium per flask. Factor 6, however, could not be controlled. Although caudal fins usually yielded the desired fibroblastic growth, scale explants commonly seeded mixed monolayer growth consisting of both fibroblast and epithelial cells, the latter often comprising the majority of the cells. The difference in success rates for karyotyping from scale-initiated versus fin-initiated cultures might be attributable to this factor.

The technique as described is simple to perform and does not require much experience. Despite being more costly and time consuming than direct chromosome preparation methods (ca. $8, 20 days per karyotype versus around $2, 2–4 hr per karyotype) and requiring special equipment (inverted microscope, incubator, and laminar flow hood), monolayer culture should prove a useful tool for characterization of karyotypes from small fishes (such as many cyprinids). In addition, chromosome banding (e.g., G- or C-banding and NOR-staining), highly promising yet almost nonexistent in fish cytogenetics, should be greatly facilitated by such cell culture systems.

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EVIDENCE FOR MULTIPLE SEX CHROMOSOMES IN THE FRESHWATER GOBY, *GOBIONELLUS SHUFELDTI* (PISCES: GOBIIDAE).

Heteromorphic sex chromosomes have been observed in very few fish species. Of the 810 species of osteichthyan fishes listed by Sola et al. (1981), there is evidence of heterogamety for only 29. In fishes with intersexual chromosomal polymorphism, sex chromosomes usually cannot be distinguished from autosomes. Their existence is frequently inferred from sexual differences of general karyotype composition (e.g., male has 19 acrocentric and 29 metacentric chromosomes, while the female has 18 and 30, respectively). Less frequently, heterogamety has been determined by observing atypical meiotic bivalent associations or recognizing two distinct haploid complements during Meiosis II (Gold, 1979).

Sex determination in the aforementioned 29 species has most often been inferred to be XX:XY, WZ:ZZ or XX:XO. Recently, multiple sex chromosomes have been reported in isolated species of seven families. An $X_1X_1X_2X_2$:$X_1X_1X_2$ system was proposed by Rishi (1976) for *Callichrous bimaculatus* (Siluridae). Filho et al. (1980) suggested a ZZ:ZW_1W_2 system for *Apereiodon affinis* (Parodontidae). All other species for which multiple sex chromosome systems have been described are believed to have an $X_1X_1X_2X_2$:X_1X_2Y mechanism (Filho et al., 1980; Levin and Foster, 1972; Murofishi et al., 1980; Thorgaard, 1978; and Uyeno and Miller, 1971, 1972)—males have one less chromosome yet equal arm numbers compared with females. Males in these species consistently have one more biarmed element than females. This suggests that each species experienced a fusion of an autosome and the Y chromosome that incorporated an autosomal pair into the sex-determining mechanism. Additional evidence for this mechanism in *Megupsilon aporus* (the cyprinodontid species of Uyeno and Miller, 1971), *Allodontichthys hubbsi* (the goodeid species of Uyeno and Miller, 1972) and *Garmanella pulchra* (Levin and Foster, 1972) was found in the formation of a trivalent during late spermatogonial prophase. In *Stephanolepis cirrhifer* (Balistidae), this system was confirmed by the presence of two karyotypes in secondary spermatocytes—one with 17 acrocentric and the other with 15 acrocentric and one metacentric chromosomes (Murofishi et al., 1980). The heteromorphic sex chromosomes are indistinguishable from autosomes in all of these cases of presumed multiple sex chromosomes, except for the Y chromosomes in *Garmanella, Megupsilon* and *S. cirrhifer.*

I am reporting the occurrence of multiple sex chromosomes in *Gobionellus shufeldti.* Evidence was obtained from mitotic and meiotic chromosome spreads for sex determination by the $X_1X_1X_2X_2$:X_1X_2Y model.

Results and discussion.—Twelve specimens of *G. shufeldti* from Irish Bayou in New Orleans, LA were karyotyped by using gill epithelium and testes according to methods developed by Kligerman and Bloom (1977) and modified by J. R. Gold (pers. comm.). Voucher specimens were deposited in the Texas Natural History Collection (TNHC 11549). Both mitotic and meiotic chromosome spreads were obtained. Eighty-nine