JOB PROGRESS REPORT

State: California

Project Number: E-W-4 Project Title: Endangered, Threatened and Rare Wildlife

Job Number: V-13.2 Job Title: California Condor Study to Develop Sexing Techniques

Period Covered: October 1, 1979 - September 30, 1980 Job Type: Survey and Inventory

SUMMARY:

A tissue culture procedure for karyotype analysis of feather pulp of vultures has been developed. Culture of feather pulp provides improved chromosome morphology, increased numbers of cells blocked in metaphase, and a reserve supply of growing tissue to insure successful chromosome preparations. A long-term study of repeated laparoscopic examination of Turkey Vultures was carried out to assess the extent of surgical trauma and healing time required after sexing birds by laparoscopy. Turkey Vultures healed completely within 8 days without evidence of visceral adhesions or inflamation of air sacs. The extensive connective and adipose tissue surrounding the viscera of vultures made observation of the gonads difficult. Successful viewing of the gonad during repeated laparoscopy of the same 7 vultures was accomplished on only 45% of attempts.

OBJECTIVES:

1. Refine technique of laparoscopy with respect to closure methods, estimation of surgical trauma, and recovery of air sac membranes and muscle tissue.

2. Develop tissue culture techniques for feather pulp of birds in order to improve the yield and staining of chromosomes as a method for sexing vultures or condors.

PROCEDURES:

The study was conducted by D. Michael Fry under a Department contract to the University of California, Davis. Captive Turkey Vultures maintained at Davis were sexed by laparoscopy repeatedly during the spring and summer to monitor the extent of surgical trauma and recovery of air sacs and flank muscles. Tissue cultures of explants and dispersed cells from feather pulp were attempted. Facilities for housing Turkey Vultures on the UC Davis campus were improved.

FINDINGS:

Study findings are included in the appended report:

Fry, D.M., and C.K. Toone. 1980. California Condor sexing technique study: improvements in laparoscopy and tissue culture for chromosomes. Job Progress Reprt, Job V-13.2, Endangered Wildlife Program, E-W-4. California Department of Fish and Game, Nongame Wildlife Investigations. 9 pp.

ANALYSIS:

Job objectives are being met.

RECOMMENDATIONS:

Four recommendations are included in the appended report.

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State of California
THE RESOURCES AGENCY
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CALIFORNIA CONDOR SEXING TECHNIQUE STUDY: IMPROVEMENTS IN LAPAROSCOPY AND TISSUE CULTURE FOR CHROMOSOMES[1/]

by

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ABSTRACT

A tissue culture procedure for karyotype analysis of feather pulp of vultures has been developed. Field collection of feather shafts is possible because shafts may be stored 24 hours at 0°C before culturing and adequate growth is still obtained. Culture of feather pulp provides improved chromosome morphology, increased numbers of cells blocked in metaphase, and a reserve supply of growing tissue to insure successful chromosome preparations. A protocol is provided which combines feather pulp squash procedures with tissue culture in a effort to utilize the shorter time required for feather pulp squashes (24-48 hrs.) and have the more reliable tissue culture method as a backup and confirmation of the sex of the bird.

A long term study of repeated laparoscopic examination of Turkey Vultures was carried out to assess the extent of surgical trauma and healing time required after sexing birds by laparoscopy. Turkey Vultures heal completely within 8 days without evidence of visceral adhesions or inflammation of air sacs. The extensive connective and adipose tissue surrounding the viscera of vultures made observation of the gonads difficult and successful visualization of the gonad during repeated laparoscopy of the same 7 vultures was accomplished on only 45% of attempts.

1/ Supported by Endangered Wildlife Program, E-W-4, Nongame Wildlife Investigations, California Department of Fish and Game, Sacramento. Job Final Report, Job V-13.2 (December 1980).

Recommendations

1) Tissue culture of feather pulp should be utilized whenever possible to maximize the chances of success of determining the sex of the bird.

2) Two feathers should be taken and cultured separately to insure success.

3) A portion of the pulp of one pin-feather should be used immediately for a feather pulp chromosome preparation and the balance saved for culture. This will minimize the time required for sexing if the immediate preparation is successful.

4) If the sex of the bird must be known within 24 hours, laparoscopy must be employed. No difficulties have been encountered with laparoscopy without anesthesia. This study has not identified any long term damage caused by laparoscopy. The gonads of vultures are difficult to locate by laparoscopy because of the extensive connective and adipose tissue which makes success with this procedure uncertain.

Introduction

The methods which can be used to determine the sex of a monomorphic bird are laparoscopy, excretory steroid hormone analysis, and karyotype analysis. Excretory steroid hormone analysis is non-invasive and gives much information about a bird's breeding condition, but is unable to differentiate sexes of juvenile vultures and can be used with certainty for sexing adults only during the breeding season when hormone levels are high and there are distinct differences betweens males and females(3). Laparoscopy and karyotype analysis are both positive methods but not without drawbacks. Surgical injury and the possibility of self-inflicted injury during recovery from anesthesia are the two primary dangers of laparoscopy. Laparoscopy of vultures can be performed without anesthesia which eliminates some risk during recovery(3). Part of this study is designed to assess the extent of surgical injury, the possible complications which might result from laparoscopy, and the time required for the healing of incisions after laparoscopy.

The major complications of karyotype analysis are time and reproducibility of the technique. Feather pulp squashes require 24 to 74 hours to obtain photographs for positive identification(4). If there are only a few metaphase figures, many hours must be spent scanning slides. Occasionally, a feather squash preparation will yield no useable metaphase figures. Tissue culture of feather pulp does not shorten the time required but tissue culture has markedly improved the chromosome morphology of the metaphase spreads and staining properties of the preparations. A combination of feather pulp squash and tissue culture now provides the most positive identification of sex with the least danger to the bird.

Purpose

A) Laparoscopy: 1) to make an appraisal of the surgical trauma to skin, muscle, air sacs and vicera of turkey vultures incurred during laparoscopy; 2) to determine the time for recovery of incision and regrowth of flank feathers.

B) Karyotype Analysis: 1) to develop tissue culture methods for feather pulp of vultures; 2) to grow feather pulp in culture and obtain a more uniform cell population for chromosome spreads; 3) to improve staining of chromosomes so that sexing of vultures is routine and reliable; 4) to develop a protocol with a very low failure rate for karyotype sexing.

Methods

A) Laparoscopy

Laparoscopy was repeatedly performed on seven turkey vultures in captivity at UC Davis at intervals of 7, 8 and 15 days and at 1 and 2 months in order to estimate the extent of trauma and the healing of birds. Vultures were hooded and restrained unanesthetized by 1 or 2 assistants and laparoscopy was performed outside under simulated field conditions. The time of healing of skin incisions, extent of bruising of muscle, trauma to air sacs and connective tissue, possible trauma to vicera and the possible adhesions of internal tissues were studied by direct observations with a 2.6mm Storz arthroscope.

Laparoscopic photography was attempted but without success as the available light source was inadequate.

B) Tissue Culture of Feather Pulp

Feather pulp was obtained from remiges or retrices of Turkey Vultures or chickens. All lengths of feathers from newly emerged pin feathers to feathers 90% of final size were cultured with very little difference in cell yield or growth rate. Birds were restrained by an assistant and the feather pulled straight out without twisting. The proximal end of the shaft was rinsed with a squirt of 70% ethanol from a plastic wash bottle and the proximal 2cm of the shaft was cut off with sterile scissors directly into a sterile test tube and capped. Feather shafts were cultured immediately or stored up to 24 hours before culture. Feather pulp was finely minced and the cells dissociated with a solution containing collagenase (300-400 units/ml Type V, Sigma Chemical Co.), hyaluronidase (135 NF units/ml Type I-S from Clostridium histolyticum, Sigma Chemical Co.), and 1.0% bovine serum albumen in a sterile saline solution or Hank's Buffered Salt Solution[6] for 20-25 minutes at 37°C with repeated pippeting with a Pasteur pippet to break up pieces of tissue. Dispersed cells were spun at 1000xg for 5 minutes, resuspended in tissue culture medium and plated into plastic tissue culture dishes or onto collagen coated cover slips (1 mg/ml calf skin collagen in water, autoclaved, pippetted onto cover slips, excess removed and cover slips air dried). Five tissue culture media were investigated in combination with four different sera and chick embryo extract. Primary cultures were fixed and stained after 1-7 days or subcultured and fixed after limited growth of the subculture. Subcultures were obtained from confluent primary cultures trypsinized briefly to disperse cells (0.5 mg/ml trypsin (Difco. 1:250) in Hanks Buffered Salt Solution), centrifuged at 1000xg for 5 minutes, and resuspended and innoculated in complete culture tissue medium. Divisions of cultured cells were partially synchronized by delaying media changes or by subculturing. The extent and timing of synchrony of divisions was determined by adding colcemid at known intervals after media change. Colcemid (GIBCO) was employed at 0.1-0.2 micrograms/ml medium in order to block mitosis.

The optimum concentration of hypotonic solutions and the time to adequately spread mitotic figures were determined for feather pulp squashes, primary cultures and subcultures. Cultures were fixed in 3:1 methanol:acetic acid and air dried, dried on a hot plate at 65°C, or flame dried. Dried cultures were stained in 2% stock Giemsa (0.8 grams Giemsa stain in 100 ml 1:1 methanol:glycerol) in water for 10-30 minutes, air dried and mounted with Permount or immersion oil (for plastic tissue culture dishes). Metaphase figures were photographed with Kodak Technical Pan film 2415 and developed for maximum contrast.

Results

A) Laparoscopy

Recovery of tissue: An incision of approximately 2 mm length was made through the skin and intercostal or obliquus abdominis muscles with a #11 scalpel blade prior to insertion of the laparoscope. When the laparoscope was removed the skin was swabed with a damp sterile gauze pad moistened with 70%

ethanol and dusted with Furacin (nitrofurazone) powder. Skin incisions were closed with sutures only if the incision was longer than 3 mm. Healing of the skin incision was rapid. The small scab which formed over the incision usually fell off within 6 days and the site of incision was rarely visible after 8 days. No infection was observed. On one occasion a feather follicle was cut and the shaft grew into the incision creating local inflamation. The pin feather was pulled a second time and regrowth to the exterior occured normally.

Full extension of the left leg pulled the sartorius muscle caudal to the paralumbar fossa and prevented cutting of this muscle. If the sartorius muscle was not cut very little hemorrhage occured when penetrating the abdominal wall. Internal tissues were examined by laparoscopy 7,8 or 15 days after an initial laparoscopic examination. The site of penetration of the air sac was not observable in any bird (18 observations) and discoloration due to hemorrhage within connective tissue was observed on only one occasion at 8 days. Fluid accumulation within or inflamation of the abdominal air sac was never observed although repeated trauma to the air sac may induce such responses (Dr. M. Fowler, pers. comm.) Trauma induced adhesions of air sacs and vicera were never observed although no birds were sacrificed and necropsied which would have allowed a more thorough examination. Care was taken not to induce more trauma than absolutely necessary during observation. Although no adhesions were found in turkey vultures the difficulty of observing adhesions by laparoscopy can not be overlooked.

The vultures had much connective and adipose tissue surrounding the vicera and air sacs compared to other large raptors such as Red-tailed Hawks. The membranes were opalescent and vacuolated, giving a foamy appearance to the adipose tissue when viewed with the arthroscope. The extent of the connective tissue made observation of the gonad difficult and the success of locating gonads in birds done repeatedly was only 45% (17 of 38 observations). During August, September and October, lipid deposition was observed and the color of the connective tissue changed from opalescent gray to yellowish.

Regrowth of flank feathers: The growth of flank feathers with black pigmentation markedly obscured the underlying musculature and the margin of the sartorius muscle could not be distinguished. Palpation of the ribs and paralumbar fossa was required in order to locate the correct insertion point. Flank feathers were totally regrown within 30 days.

Summary: Laparoscopy was performed at intervals as often as 7 days without observing any prolonged trauma, fluid in air sacs, or adhesions. Skin incisions were healed within 6 days and the site of incision was not usually visable after 8 days. The extensive connective tissue of vultures made observation of the gonads difficult and gonads frequently could not be visualized.

B) Tissue Culture of Feather Pulp

Storage of Tissue Prior to Culture: Feather shafts of Turkey Vultures were obtained sterily and stored 4 hours or 24 hours in: A) Hanks Balance Salt Solution; B) Medium NCTC 135[2], or; C) dry. Shafts were kept at 0°C or 20°C and cultured as described below. Growth of the cultures was assessed at 48

hours.

Storage of shafts for 4 hours at 0°C had minimal detrimental effect on culture growth but all cultures of shafts stored at 20°C had lower cell populations and increased noncellular debris. Storage of shafts in sterile Hanks Balanced Salt Solution yielded cultures with the highest cell population and only slightly lower cell populations in cultures of shafts stored dry or in Medium NCTC 135. Holding of feather shafts for 24 hours, however, resulted in marked differences between storage conditions. The best survival and subsequent growth was in shafts held at 0°C in medium NCTC 135. The cell population was reduced about 50% from 4 hours of storage but the cultures grew vigorously and with little extracellular debris. Storage at 20°C reduced the cell population to less than 10% of 0°C storage. Adequate but slower cell growth was obtained from shafts stored in Hanks Balanced Salt Solution or stored dry at 0°C. Almost no growth was obtained from shafts stored dry at 20°C.

Tissue culture media: Cell growth was highly dependent on the medium employed. Media were selected for maximum growth and division rate of cells. Mixtures of chemically defined media (Chromosome Medium 1A (GIBCO), McCoy's 5a Medium[7,8], Eagles Minimal Essential Medium[1], NCTC 135[2], and Ham's Nutrient Mixture F12[5]) plus 5-10% serum (chicken, calf, fetile calf, or horse) and 2-10% chick embryo extract were assessed by plating dispersed cells into 35mm plastic petri dishes and observing growth for 2-4 days. The most successful medium for both chicken and turkey vulture feather pulp was 78% NCTC 135, 10% horse serum, 10% chick embryo extract, and 2% penicillin streptomycin solution (final concentration 100 units per ml penicillin, 100 micrograms per ml streptomycin(GIBCO)). This medium was used for all storage, synchrony and subculture studies.

Synchronization of cell divisions: Medium NCTC 135 contains more defined constituents than any of the other media indicating that specific nutrients which were required for rapid growth and division may be deficient in the other defined media. The depletion of essential components from the medium was used as a method to partially synchronize cell divisions and increase the number of metaphase figures in a given cell culture. The rate of cell division decreased 48-72 hours after a medium change and an increased fraction of cells could be blocked in metaphase 17-21 hours after a medium change indicating that fresh medium probably removed a block to synthesis of components necessary before cell division could occur. Cells entering mitosis were blocked with colcemid and the peak of mitotic activity was determined by adding colcemid to cultures at known intervals after medium change.

Hypotonic solutions: The chromosomes of dividing cells blocked in metaphase by colcemid were preserved by swelling the cells in a hypotonic medium, fixing, and air drying to spread the mitotic figures. Cultured cells responded differently from feather pulp squashes in their response to colcemid and hypotonic saline. Primary cultures after 24-48 hours of culture responded much like feather pulp squashes. Hypotonic saline treatment of 0.75% sodium citrate or 0.45% NaCl for 40 min. or 1 hr. resulted in optimum cell preparations. Culturing for longer periods and subculturing resulted in more delicate cells which required less harsh treatment for adequate swelling. The most satisfactory hypotonic solution for subcultures was a dilution of complete

culture tissue medium with 3 or 4 volumes of 37°C water for 5-15 minutes. When dilutions of media were used as hypotonic saline for primary cultures 8:1-15:1 dilutions were found to be optimum.

Subcultures: Secondary cultures of feather pulp cells provided 3 distinct advantages over primary cultures: 1) increased of number of dishes; 2) partial synchrony of cell division, 3) more even staining of chromosome preparations. Primary cultures were grown to confluence, trypsinized and subcultured onto collagen coated cover slips or plastic petri dishes at a reduced cell population. Growing the cell cultures to confluence inhibited cell divisions and the cultures could be partially synchronized by diluting the cells and replating in fresh medium. Fresh subcultures had a high mitotic index at approximately 20 hr. after innoculation.

Subcultures exhibited more even swelling of cells and more even staining of chromosomes than either feather pulp squashes or primary cell cultures. We attribute the more constant response to the selection of a homogeneous cell population suited to the conditions of culture. Those cells best suited to the medium and growth conditions probably grew faster and became the dominant cell type. As a result cultures become more homogenous with longer culturing.

The karyotype of a female turkey vulture is shown in figure 1 along with the original metaphase figure. The morphology of the chromosomes is much more distinct than the vast majority of preparations from feather pulp and the 2N number of 80 is identical to the diploid number of the King Vulture and Andean Condor reported by Takagi and Sasaki[9]. The improved chromosome morphology provided by tissue culture has allowed the identification of many individual chromosomes. The W chromosome (female sex chromosome) is identifiable as a small metacentric. The chromosome arm morphology could not be determined from feather pulp squash preparations.

Summary of Tissue Culture: A growing remige or retrice is utilized for culture. Feather shafts may be stored sterile for 24 hours at 0°C in Medium NCTC 135 before culturing. Medium NCTC 135 plus 10% horse serum, 10% chick embryo extract and penicillin-streptomycin yields the best growth for chicken or Turkey Vulture feather pulp. Partial synchrony of cell divisions is obtained by growing the cells to confluence, subculturing and blocking mitosis with colcemid at 16-20 hours after subculturing. Dilution of medium with 37°C water yields controlled swelling. Cells are fixed in 3:1 methanol:acetic acid for 1 hour, quickly dried and stained with Giemsa.

C) Protocol for sexing of vultures by feather pulp squashes and tissue culture.

1) Pull two feathers if possible. Rinse with ethanol and place the proximal 2cm of feather shafts in two sterile test tubes. 2) Remove a portion of pulp of 1 feather shaft immediately for a feather pulp squash. Place into 0.75% sodium citrate for 1 hour at 37° and fix in 3:1 methanol:acetic acid. Retain the balance of the 2 shafts for culture. Store in NCTC 135 at 0°C for up to 24 hours 3) Culture the 2 feather shafts individually to minimize chances of contamination: 4) Arrest mitosis in one primary culture with colcemid at 20 hours after innoculation. Save the remaining dishes for

subcultures 5) Subculture the dishes when cell confluence is obtained, usually within 4 or 5 days. Prepare metaphase preparations from subcultures on day 6. With very favorable feather pulp squashes and a Polaroid microscope camera feather shaft sexing can be done within 24 hours. Primary cultures can be used after 20 hours if sufficient cells have been innoculated. Innoculating multiple culture plates and culturing two feather shafts indepentently should insure successful chromosome preparations.

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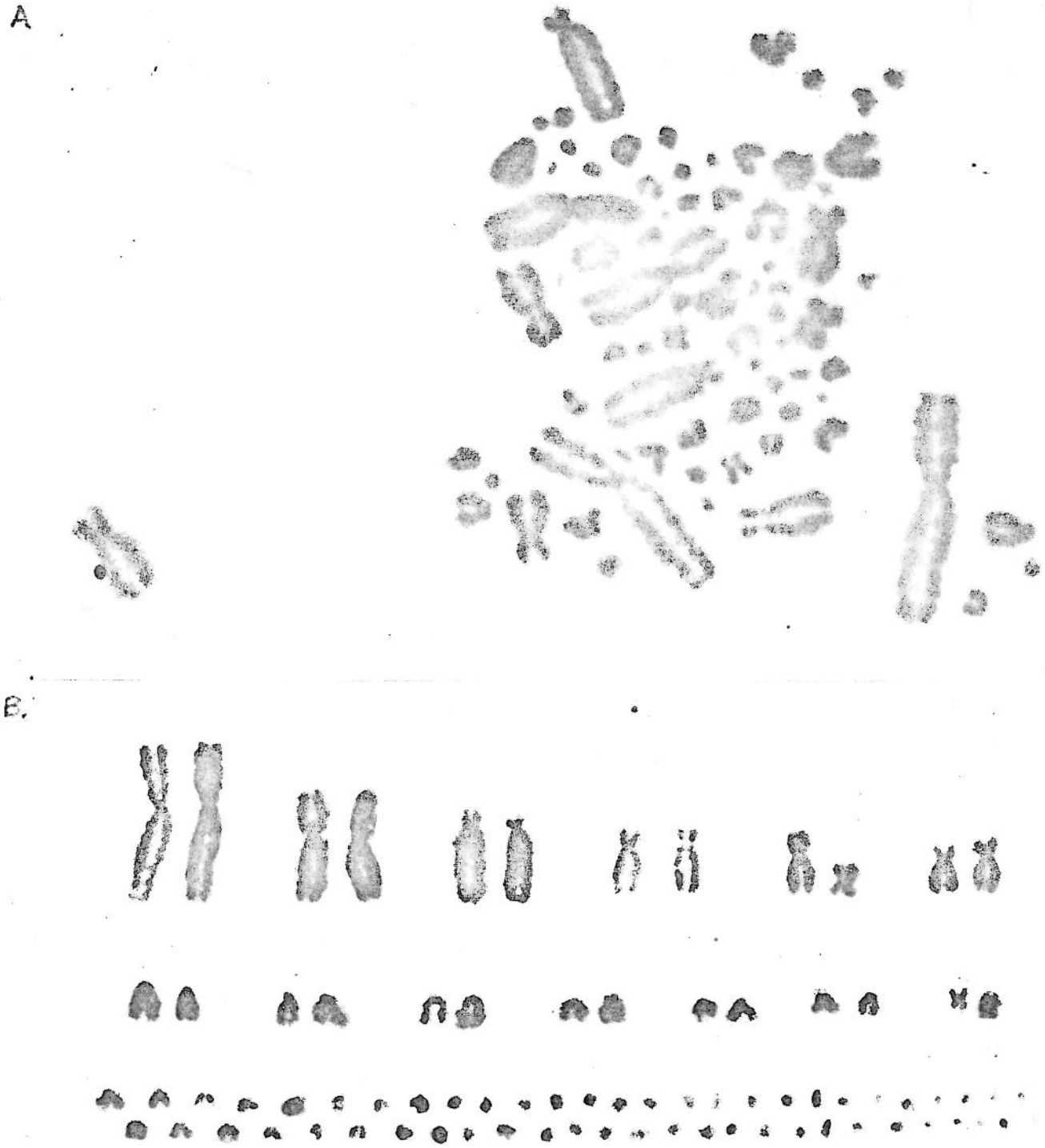

Figure Legends

Figure 1. A) Metaphase spread of tissue culture cell of a female Turkey Vulture blocked during cell division with 0.1 microgram/ml colcemid. The W chromosome is identifiable as the unpaired metacentric. 4000x.

B) Karyogram of female Turkey Vulture. The 2n number of 80 and the chromosome morphology is identical to the karyograms of King Vulture and Andean Condor described by Takagi and Sasaki[9].