A simple animal chromosome technique for use in field laboratories

by

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ABSTRACT

A chromosome preparation technique has been developed over several years for use primarily with birds. The technique is similar to others in general use, being based essentially on the methods of Ohno and Shoffner. Modifications were developed specifically for use under "field laboratory" conditions, with a minimum of steps and apparatus, including the option of using biopsy material without cell culture. The technique has been successfully used on mammals, reptiles and fishes, with minor adjustments. It has been successfully field-tested in Africa and South America.

The procedure involves Colcemid or colchicine administration *in vivo*, *in vitro*, or both. Embryonic or young material is preferred. After hypotonic treatment, material is fixed in acetic acid solution and squashed on a clean slide. The coverslip is removed after freeze-fixation in a methanol – dry-ice bath (dry ice may be made *in situ* from a CO_2 cylinder). The preparations may then be stained with Giemsa after acid hydrolysis (standard stain), and permanently mounted. Alternatively, preparations may be banded following aging/hardening procedures: the technique seems to be compatible with several different banding methods including trypsin G- and $Ba(OH)_2$ C-banding.

The method is useful for sex determination in some vertebrates, and for testing cytogenetic and systematic hypotheses.

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1 INTRODUCTION

Chromosomes, the basic carriers of the hereditary "blueprint", have been studied since the late 19th century. Only rather recently, however, have techniques improved sufficiently to apply these studies in investigations of an organism's biology. Birds have been among the more difficult of karyological subjects despite the early successes of a few investigators like Yamashina (1943, 1944), who used histological sectioning techniques.

Chromosome banding techniques were introduced in the 70s for mammalian material (Caspersson *et al.* 1970, Seabright 1971, Sumner *et al.* 1971) and rapidly assumed an important role in karyotype analysis. Several types of banding have been developed, but the principle is the same: chromosomes around early metaphase are differentially denatured, then stained, yielding a chromosome-specific transverse-banded pattern (usually from one or two to seven or more bands). Morphologically similar chromosome pairs may often be differentiated by their characteristic band patterns. Fine-structure analysis of chromosomal rearrangements and other phenomena has thereby been made possible. These techniques have been applied to other organisms with only moderate success to date (e.g. Stefos and Arrighi 1971, Wang and Shoffner 1973, Takagi and Sasaki 1974, for birds; and Howell and Bloom 1973, Ojima and Ueda 1978, for fishes).

In the mid-60s I became interested in developing a simplified and generalised chromosome preparation technique for animals, usable under relatively primitive field laboratory conditions. Ideally, such a method would fulfil the following requirements:

1) Technical simplicity, allowing use in the field with minimal facilities, apparatus and chemical supplies; 2) general applicability to birds and other animals; 3) chemical simplicity allowing easy modification to special staining or other procedures at any stage;

4) immediate as well as longterm availability of preparations for analysis; 5) optional use of harmless biopsy material; 6) consistently good quality of preparations.

These considerations ruled out all techniques using sectioning, cell culture, differential centrifugation and other procedures requiring tight control of chemical/physical conditions.

A colchicine – hypotonic – squash sequence was eventually adopted and adapted (Jensen 1967). While not entirely fulfilling all the above requirements, the present version of this method produces consistently acceptable results with birds and other vertebrates.

Modern colchicine – hypotonic – squash/drying methods date from Tjio and Levan's historic 1956 paper on the chromosomes of man. Subsequently many modifications were introduced; examples may be mentioned for birds (van Brink 1959, Ohno 1961, Rothfels *et al.* 1963, Ohno *et al.* 1964, Krishan *et al.* 1965, Krishan and Shoffner 1966); for reptiles (Becak *et al.* 1964); for amphibians (Becak 1968); and for fishes (Simon 1964, Ohno *et al.* 1965, Denton 1973).

The method given in detail here is based on squash techniques developed by the groups of Dr. Susumu Ohno at the City of Hope Medical Center, Duarte, California, and Dr. Robert N. Shoffner at the University of Minnesota, St. Paul. I have incorporated various modifications, mostly for simplicity, versatility and improved resolution. These procedures were developed over a 15-year period, and have been successfully field-tested in South America and southern Africa. Further discussion of the significance and chemical rationale of the procedures may be found in Darlington and La Cour's (1962) excellent, if dated, chromosome text, in Schwarzacher and Wolf (1974) and, for banding, in Kato and Moriwaki (1972), among others.

2 METHODS AND MATERIALS

2.1 Basic squash sequence

2.1.1 Sacrifice method (for adult birds and other vertebrates)

Step 1: Animals are selected as young as possible. A 0,5% aqueous colchicine solution (Appendix II) is injected intraperitoneally. See Table 1 and Figure 1a for suggested dosages.

Step 2: After 50-90 minutes (see Table 1) the animal is decapitated and pieces of spleen, liver, kidney and/or testis are excised. The tissue is then cut up into approximately 1 mm^3 pieces and dropped into 0,45% sodium citrate for 10-20 minutes (see Table 1). For testis especially, colchicine may be added as in step 2c for embryos. Note recommended temperatures.

Step 3: The hypotonic solution is carefully decanted and replaced with 50% acetic acid for 30 minutes. Fixative should be at room temperature, i.e. same temperature as material at end of hypotonic treatment.

Step 4: Cleaned slides and coverslips (see Appendix II) are used for squashing. Taking the utmost care to avoid the introduction of any form of lipid onto the slide, a drop of fixative is placed on it along with a piece of tissue. The blunt flattened end of a diamond pencil or similar chemically stable implement is then used to tap the tissue gently into suspension. A little more fixative may be added if necessary to dilute the suspension. The slide is tilted slightly to allow a drop of suspension to roll away from the macerated tissue. A small droplet of this separated suspension is then transferred with the end of the tapping implement to a second cleaned slide. With care it is possible to transfer essentially a cell suspension to the second slide (i.e. very little acellular debris).

Step 5: A 24 x 40 mm No. 1 cleaned coverslip is placed over the droplet. The slide is inverted on bibulous paper and tapped with a non-metallic object such as a plastic-capped pencil. Excess fluid (which should be kept to a minimum, especially for bone marrow and feather-pulp, which "roll" easily) is expressed by gentle pressure. The slide is then righted and squashed with gradually maximised pressure from both thumbs on the coverslip through one or two thicknesses of bibulous paper. Sidewise movement of the coverslip must be avoided at all costs. A low working surface (about mid-thigh height) is preferable for optimal squashing.

Step 6: The slide is suitably inscribed with a diamond-point pencil, and immersed in a methanol – dry-ice bath previously prepared (see Appendix II). After at least one minute, the slide is taken out and the coverslip flipped off immediately with a razor blade. The slide is then allowed to dry.

Step 7: The slide may be immersed in absolute ethanol for 5 minutes at room temperature, especially when lipid and melanin are present. It should then be thoroughly air-dried.

At this stage, slides may be stored safely for at least a week before further processing, provided fungal attack is not a problem (in the tropics especially, it is essential to keep them in a dry atmosphere, e.g. in active, sealed dessicator jars).

2.1.2 For pulli and late-stage avian embryos

Step 1b: For chicks and nestlings, 0,5% colchicine is injected intraperitoneally (see Table 1 and Figure 1b). For large embryos (older than half the incubation period, in general), 0,05% "Colcemid" (CIBA) may be used (see Table 1 for dosage, and Appendices I and II for Colcemid recipe).

Step 2b: After 45-50 minutes the bird is decapitated and spleen, liver, gonads and kidney are excised and cut into approximate 1 mm cubes in 0,45% sodium

TABLE 1: Chromosome treatment protocols for various vertebrate tissues

Animal/Tissue	Pretreatment – Mitotic Arrest ml/mass/time	Hypotonic (0,45% Na-citrate) time/temperature		Hydrolysis (1N HCl) time/temp	Standard Giemsa Stain (Rack)
	0,5% colchicine:				
Birds, ad., somatic (>1 kg)	* 0,6 ml/kg, 50-60 min	10-20 min, 40°C↘RT	50% Acetic Acid, RT, 30 mins minimum, followed by squashing and freezing in methanol–dry ice, coverslip removal, drying, 5 min immersion in ethanol (optional)	10 min, 58°C-60°C	10-15 min
Birds, ad., somatic (<500 g)	* 0,1 ml/100g, 50 min	10-20 min, 40°C↘RT		10 min, 58°C-60°C	10-15 min
Birds, ad., testis	as above, by mass	15-20 min, 40°C↘RT †		8 min, 56°C-58°C	5-10 min
Birds, pulli (all tissues)	** 0,2 ml/25g, 45-50 min	15 min, 40°C↘RT		8 min, 56°C-60°C	6-10 min
Lizards, somatic and gonad	0,1 ml/10g, 60 min (minim. 0,05ml)	15-20 min, RT †		8 (testis)-10 min, 58°C-60°C	10 min
Fishes, somatic and gonad	0,2 ml/10g, 60-90 min (minim. 0,1ml)	20 min, RT †		8 (testis)-10 min, 58°C-60°C	10 min
	0,05% Colcemid:				
Birds, late embryos/young pulli	0,2 ml/50g egg, 50 min (minim. 0,1ml)				
Birds, early embryos	0,1 ml/50g egg, 40-45 min (minim. 0,05 ml)	15-20 min, 40°C↘RT		6-10 min, 56°C-58°C	5-10 min
Birds, feather pulp (live method)	0,05ml/20g, max 1 ml/kg, 50 min	20-30 min, 38°C↓RT †		6- 8 min, 56°C-58°C	2- 5 min
Birds, feather pulp (live – no risk)	no pretreatment	15-20 min, 38°C↘RT †		6 min, 56°C-58°C	2- 3 min
Lizards, eggs	††0,05-0,1 ml for eggs 1-5g, 50 min	30-40 min, 38°C↓RT †		6 min, 56°C-58°C	2- 3 min
Fishes, eggs	††0,1 ml/10 ml water in watchglass, 60 min	20 min, ††°C↓RT †		6- 8 min, 56°C-58°C	5- 6 min
	(or use colchicine 0,5% 0,05 ml)	20 min, ††°C↓RT †		6- 8 min, 56°C-58°C	5-10 min

* See adult bird dosage curve, Figure 1a.
** See chick dosage curve, Figure 1b.
† 0,5% colchicine added optionally (see text), 0,1 ml/50 ml hypotonic solution.
↘ Allowed to cool over the indicated period to RT (room temperature).
↓ Incubated at the starting temperature, allowed to cool to RT over last 5-10 minutes.
†† Pretreatment and hypotonic treatment should be carried out at the appropriate incubation temperature for the species' eggs.

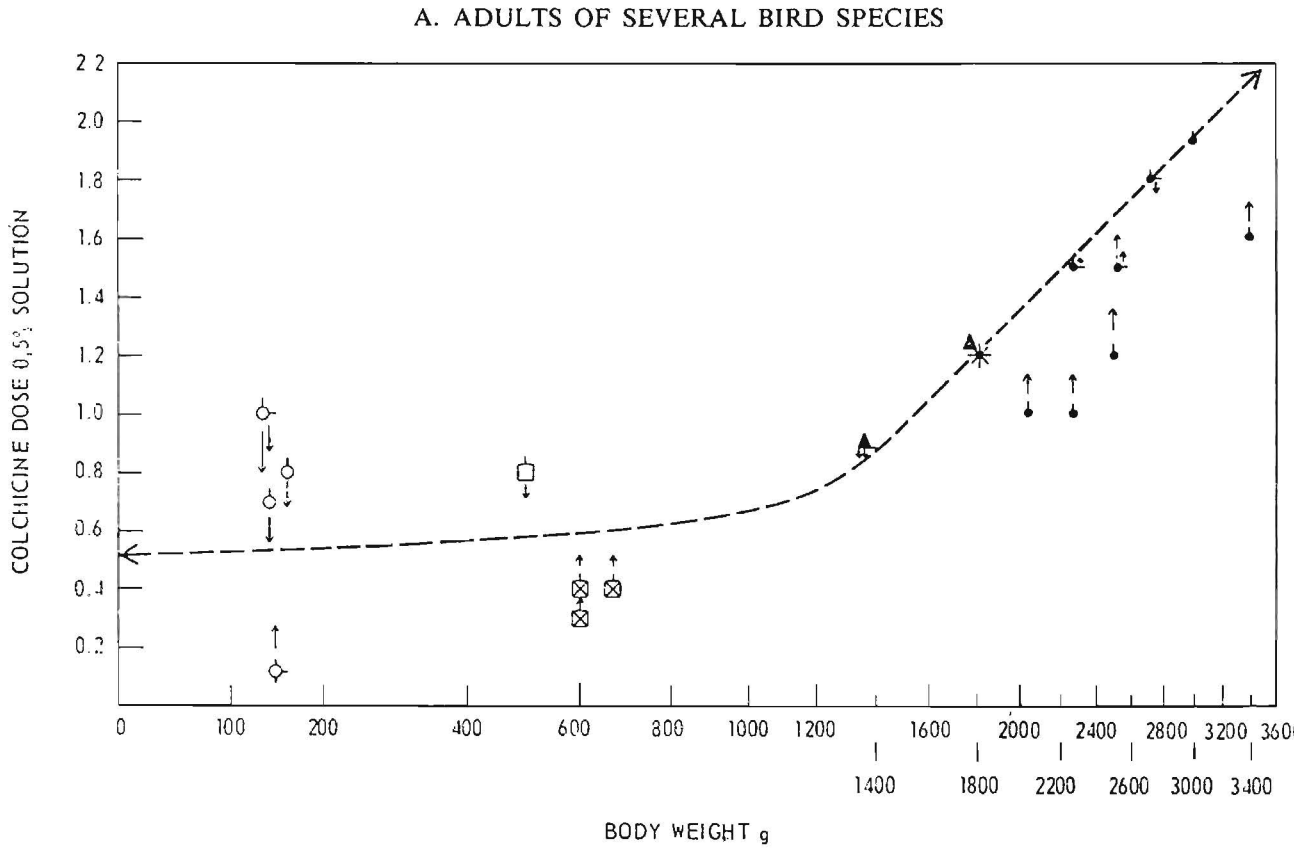

FIGURE 1A: Interpolated semi-logarithmic plot of colchicine dose vs. body weight for adult birds of various Galliform species. Weights on log. axis.

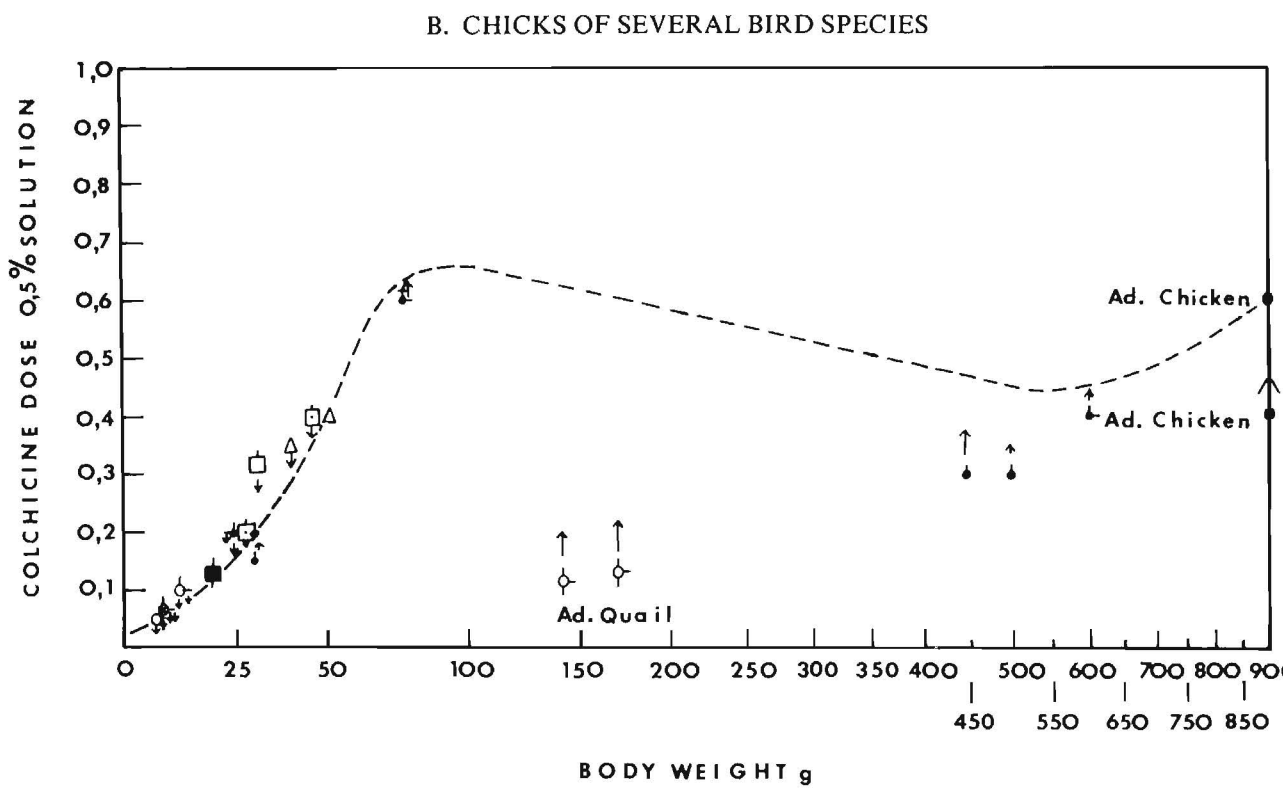

FIGURE: 2B: Interpolated semi-logarithmic plot of colchicine dose vs. body weight for chicks 1–40 days old, from several different species. Weights are on log. axis. (Some adult plots from fig. 1A are included for comparison.)

Explanation of Symbols

● = Chicken
○ = Japanese quail
⊗ = Chicken × quail hybrid
⊠ = Golden × Amherst pheasant hybrid
▲ = Guineafowl
□ = Chukar partridge
■ = Ringneck pheasant
△ = Snow partridge
⊡ = Tinamou
| = One individual;
♂ = Two quail; etc.

Broken line represents suggested optimal dosage – weight relationship. Arrows at symbols represent over (⬇) and under (⬆) dosage respectively.

citrate at 37°-40°C. After 15-20 minutes during which the hypotonic bath is allowed to cool to room temperature, the fluid is decanted and the tissue handled as described for adults, steps 3 and 4. The cell suspension resulting from step 4 is then squashed as described in steps 5 and 6. It may also be air-dried or flame-dried (Rothfels and Siminovitch 1958) after transfer to 3:1 methanol – glacial acetic fixative, but in my experience this method yields less satisfactory results with non-mammals. In either case, an ethanol bath may be used to conclude the sequence (step 7).

2.1.3 For avian early embryos (mid-incubation or less) and small eggs of other vertebrates

Step 1c: From embryos large enough, liver, gonads and spleen are good sources of material. Injection of Colcemid 0,05% solution (Appendix II) is via the allantois (see Table 1 for dosage and duration). For small embryos, Colcemid is injected into the amniotic cavity. The embryo may either be removed and incubated in physiological saline or treated in the egg. The whole embryo, or (if large enough) the midbody or brain, may be used for chromosome preparations. See Table 1 for recommended times and temperatures (the latter should be at or near the incubation temperature for the respective eggs).

Step 2c: The tissue is chopped into approximately 1 mm^3 pieces and treated in hypotonic 0,45% sodium citrate, starting at the appropriate temperature (Table 1). If the embryo died in the previous step, or just prior to it, the addition of 0,1 ml of 0,5% colchicine to 50 ml hypotonic solution, and incubation in this for 20-30 minutes at the starting temperature is recommended. This latter treatment may also be used advantageously with other young material, provided it has not already been "over-colchicined". Subsequent processing is as for chicks and older embryos from step 3.

2.1.4 Live method (Feather pulp of birds)

This method works only with actively growing feathers, preferably from young chicks and nestlings (pulli), but moult-growing feathers from adults can also be used. Birds suffer no serious damage provided primaries and rectrices (major wing and tail feathers) are not used. The younger the feather, the more active its basal pulp.

Step 1d: Inject 0,05% Colcemid (Appendix II) into a wing, 0,05 ml per 20g weight, and no more than 1,0 ml for any bird up to 1 kg. (For extremely valuable birds, or other reasons, this step may be omitted).

Step 2d: After 50 minutes, large growing feathers such as secondaries, greater wing coverts, greater tail coverts, are plucked (3-4 will give enough material usually) and feather pulp squeezed from the basal 1-4 mm of each feather by sliding forceps over that part of the shaft. Special care must be taken with dark feathers to use the clear part of the pulp, avoiding as far as possible the introduction of melanin to the preparations. If there is a choice, the lightest-coloured feathers should be used. The pulp is cut up into 1 mm^3 pieces and dropped into warm sodium citrate solution with colchicine (see Table 1). If step 1d has been omitted, incubate for 30-40 minutes at this temperature, then allow to cool 5-10 minutes. Otherwise, allow to cool to room temperature over 15-20 minutes.

Subsequent handling is just as previously given for pulli, from step 3.

2.2 Standard Giemsa staining

There are several options in standard staining. My preferred procedure is rack-staining preceded by acid hydrolysis.

Step 8: The thoroughly dry slide preparations are hydrolysed in 1 N hydrochloric acid (see Appendix II) according to the times and temperatures indicated in Table 1.

Step 9: Slides are rinsed in tap water 5 minutes, then in distilled water, and stained on a rack by flooding each slide with distilled water and adding three to four drops of Giemsa stock solution (Appendix II) per slide. Stain is mixed with the water on the slides by blowing with air (I use a rubber squeeze bulb and tube).

Step 10: After staining 2-15 minutes (see Table 1) the slides are rinsed with distilled water and air-dried. In humid conditions a dessicant should be used, as drying must be thorough.

Step 11: The preparations are made permanent with "Permount" (Fisher Scientific, see Appendix I), following a one minute bath in toluene. Optical resolution of the chromosomes is improved by pressing the coverslip down as flat as possible, and weighting it with small vials for 48 hours or so until the mountant is fairly well dried around the edges. "Permount" may be diluted with toluene to facilitate this.

2.3 Banding techniques

These fall in two broad categories, viz. fluorescence and bright-field banding. With the co-operation and assistance of the Human Cytogenetics Laboratory at Groote Schuur Hospital, Cape Town, my students and I tried various fluorescence-banding procedures on non-mammalian vertebrate material in 1978-1979. Most of the results were less than satisfactory, the most persistent problem being the extremely short duration of differential fluorescence (a few seconds in some cases), when bands were obtained at all.

Some indication of Q-banding was obtained in birds, frogs and fishes, using a "QFQ" staining sequence (Q-band Fluorescence with Quinacrine, in the terminology of Verma and Lubs). The slides had been processed as previously detailed up to and including step 7.

R-banding by the "RFA" (Reverse-band Fluorescence with Acridine orange) method of Verma and Lubs (1975, 1976) yielded more promising results than Q-banding with birds and fishes, though unfortunately with the same problem of extremely rapid band-fading, making photography impossible. This technique can probably be successfully modified for general vertebrate use; it yields spectacular results with human material (Verma *et al.* 1977).

I have not tried NOR (Nucleolar Organizer Region) silver staining, but Goodpasture and Bloom (1975) published a versatile method which can probably be adapted for all vertebrates.

The easiest and most consistently successful banding technique compatible with my squash preparation procedure has been C-banding. This may be regarded as an extreme form of G-banding, obtained after more drastic treatment. For birds, G-banding seems to be most successful via the trypsin digestion methods. Some indication of G-banding was attained with fish and reptile material. Additional investigation to optimise treatment protocols for these organisms is required.

The two banding methods given below are based on procedures used for cultured human cells at the Groote Schuur Human Cytogenetics Laboratory. These are in turn adapted from previously-published methods as indicated. Results are less consistent than with mammal material: the aging history of the slide after freeze-fixation and probably other yet unfathomed factors seem to be crucial.

After squashing and freeze-fixation as previously described (to step 7):

2.3.1 "GTG" method (G-bands with trypsin using Giemsa stain) adapted from Seabright (1971)

Step 8G: Age the slides for about a week in a dry environment, but try shorter and longer periods also. The process is speeded by incubation in a dry oven at 60°C overnight immediately prior to banding.

Step 9G: Prepare PBS (phosphate-buffered saline solution), 0,20% trypsin working solution and pH 6,8 buffer (see Appendices I and II). Set up two Coplin jars of PBS rinse, one of trypsin solution. The latter is critical: working solution must be freshly prepared from active, properly stored stock.

Step 10G: Dip slide into trypsin solution at room temperature (c. 22-24°C), with initial agitation to dislodge air-bubbles. Start with 30 seconds as a test immersion, and be prepared to "bracket" this "exposure" widely.

Step 11G: Remove and dip slide immediately into first PBS, then rinse more thoroughly in second PBS jar.

Step 12G: Rinse with 6,8 buffer, then stain in pH 6,8 buffered Giemsa (Appendix II), either on a rack or (preferably) in a jar, 3-5 minutes.

Step 13G: Rinse in same buffer and distilled water briefly, shake dry (I do not recommend blotting) and examine under moderate power, e.g. 400x.

Slides may be permanently mounted, if satisfactory, as previously described with "Permount" or other mountant (step 11, previous section). They may also be examined unmounted under oil. In that case later permanent mounting must be preceded by thorough de-oiling in the appropriate solvent (toluene for "Permount").

Notes: Over-trypsinised chromosomes appear bloated and empty (ghost-like), virtually unstained. Under-trypsinised slides (even-stained) can be taken through the sequence again after destaining in alcohol. Over-staining may sometimes be corrected by extra rinsing in buffer to improve band differentiation (decrease staining time for subsequent slides of the same batch). Under-staining simply requires additional staining time, but unfortunately this will not help over-trypsinised chromosomes.

G-banding typically produces light-staining centromeres, with alternating light and dark transverse bands of different size and number along the chromosome arms.

2.3.2 "CBG" Method (C-bands with barium hydroxide using Giemsa stain) adapted from Sumner *et al.* (1971)

Step 8H: Aging treatment is similar to, but not nearly as critical as, that for G-banding given above.

Step 9H: Prepare Barium hydroxide $Ba(OH)_2$, and double saline sodium citrate (2xSSC) solutions – see Appendix II. Also prepare 95% (two), 70% ethanol baths, normal saline and buffered acetone Giemsa stain solutions (these latter may be conveniently prepared during 2xSSC treatment, step 15H; see Appendix II).

Step 10H: Preheat waterbath with 2xSSC container to 60°C.

Step 11H: Rinse slides in two 95% ethanol baths.

Step 12H: Wash slides in normal saline.

Step 13H: Place slides in saturated $Ba(OH)_2$ at room temperature (c. 22-24°C) for 10 minutes.

Step 14H: Rinse in 70% alcohol, wash well in normal saline, removing all trace of $Ba(OH)_2$.

Step 15H: Place in hot 60°C 2xSSC for 3½ to 3¾ hours.

Step 16H: Wash well in hot (not over 60°C) tap water.

Step 17H: Stain flat on rack with buffered acetone Giemsa for 2-3 minutes.

Step 18H: Rinse with 6,8 buffer and distilled water, shake dry and examine. Mount permanently if satisfactory, as described previously.

Notes: If stain is too light, increase time. If too dark, decrease acetone proportion and shorten time for subsequent slides – try de-staining and restaining affected slide.

C-banding typically stains the so-called constitutive heterochromatin dark (e.g. around centromeres, most or all of bird W chromosomes and occasionally other regions, especially terminal).

2.4 Analysis and photography

For checking preparations during processing and thereby saving considerable time if processing must be modified or repeated, phase contrast optics are essential, in the magnification range 200-400x. An extremely handy field unit is the "Nikon Hand Microscope" equipped with some phase optics. This instrument is about the size of a large 35mm camera and may be used anywhere. Its major disadvantage is that slides must be inverted for viewing, and the small stage necessitates extreme care in handling "wet" slides.

Preparations are scanned at about 200x on a good compound microscope and promising metaphases examined more closely at about 400x. Co-ordinates are noted on the vernier stage so that photography under high power (1000 – 1200x) with oil immersion objective may be carried out later. Flat-field low and high-power objectives are desirable, with the latter having the best possible resolution (currently plan-apochromats). The middle range (200-400x) objectives should be phase types of good quality, e.g. fluorites, since they will be used both in phase contrast and in bright-field mode.

For photography 35mm, 6 x 6 cm and 6,5 x 9 cm formats are the most useful, along with polaroid. Better quality photomicrographs for publication are usually obtained on the 6,5 x 9 cm sheet film. I use high contrast films: Kodak Photomicrography Color Film (35 mm) 2483, and for black-and-white, various high-contrast, high-resolution, fine-grain films, developed as the manufacturers suggest for maximum resolution.

3 DISCUSSION

Chromosome preparations by the simple technique detailed in the previous sections may be carried out with minimal laboratory facilities and equipment, although chemical preparation beforehand would require standard laboratory facilities like balances and pure water, while final analyses must be done with a sophisticated compound microscope. A plastic cool-box of about 0,05 m^3 capacity (1,7 cubic feet) will take all the necessities, except a CO_2 cylinder, for several weeks' field processing. The only *in situ* indispensable requirements are a clean, firm, flat, smooth working surface and clean water.

Colchicine is pre-weighed in rubber-capped injection vials to which pure water is added when processing begins. The solution should be good for at least a few days under tropical conditions, but is discarded when cloudy. Colcemid is made up as a working, sterile solution. A thermoplastic-lined small thermos flask is suitable for the methanol-CO_2 freeze bath. Tissue samples are treated in small teflon or polypropylene beakers, and incubation when required is done at body temperature or in a water bath under field conditions.

Unfortunately, no method seems to be available for preserving animal tissue adequately in the pre-squash stages, to yield acceptable chromosome preparations later. However, the procedures given above may be interrupted safely at several stages after freeze-fixing the squash slides. Following freezing and removal of the coverslip, slides may be stored in a dry atmosphere for days or even weeks. In humid and tropical conditions, however, there is great danger of fungal attack, so slides should be further processed as soon as possible after squashing.

Permanent mounting of stained slides may likewise be delayed provided slides are stored in a dry atmosphere.

Air-drying, or heat-drying (e.g. Rothfels and Siminovitch 1958) is preferred to squashing by most authors dealing with mammals. Generally, I have found it less satisfactory for organisms with large diploid numbers and/or tougher cells (e.g. adult birds). However, drying tends to give better banding results and is therefore sometimes worth trying: it may be easily fitted into the post-fixation sequence as indicated in the section for pulli and large embryos.

Hydrolysis and some banding procedures require critical temperature control. This is liable to present problems in the field, and should be postponed if possible till access to more sophisticated facilities. A water-bath is usually the best option. The ephemeral nature of fluorescence banding of course mandates its use only with direct, immediate access to a fluorescence microscope.

Some staining techniques may be applied sequentially to the same slide, for example F-, G-, and C-banding. Unfortunately, the sequence seems to work only in one direction (at present). Standard-stained slides cannot be banded subsequently. Despite many attempts both in my laboratories and elsewhere over the years, no success has attended efforts to band old, previously stained slides. It seems that irreversible changes take place in the chromosomes during the hydrolysis and staining

phase of slide preparation. These changes may be analogous to the changes which occur during slide aging, in reverse, but might also be chemically quite different. Slide aging for banding appears to be a tricky and variable procedure; different laboratories seem to have individual peculiarities in this regard. Although some age their slides only a few days, without heat, others report excellent banding results after months of aging. Relative humidity during storage has been suggested as an important contributing factor.

Permanent mounting with "Permount" has been most successful for me: standard Giemsa-stained slides 15 years old have not suffered any noticeable loss in quality of chromosome spreads. Other mountants such as DPX ("DePex") are widely used.

Karyotype analysis in the various modes made possible through different staining has numerous applications in biology, including field-oriented studies. Among potential applications may be listed the following (several of which I have utilised): sex and sex-chromosome identification; species identification (from flies to brood parasites); systematic (taxonomic) placement; polymorphism; convergence; developmental sequences and reversed sequences; hybrid sterility; and genetic conservatism. The list only begins with these.

In conclusion, the generality of the basic method detailed in this paper should be stressed. The technique has been successfully used with minor adjustments for reptiles, birds, fishes, mammals, amphibians and even beetles! Successful "*ex vivo*" variations along the lines of the bird feather pulp method have yet to be developed for most of these groups. Hair base pulp and fish fin epithelium hold promise in this regard.

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APPENDIX I: Source list of some chemicals and materials used for the cytological procedures. In most cases these chemicals are also available from other sources.

Compound	Source
Colcemid	CIBA Pharmaceutical Co. (In S.A. Ciba-Geigy Ltd., Isando, Tvl.)
Colchicine	P grade: H.M. Chemical Co., Santa Monica, Calif.; USP grade: Fisher Scientific Co., Fairlawn, N.J.
Giemsa Stain (powder)	National Aniline Division of Allied Chemicals Corp., N.Y.
Giemsa Stain (solution)	G.T. Gurr; BDH; Fisher Scientific; Merck.
Methanol, Absolute, Reagent Grade	Baker Chemical Co., N.J.; Merck Chemicals; Protea Holdings, Ltd.
Permount	Fisher Scientific Co., Fairlawn, N.J. (obt. Protea Holdings, Ltd.)
CO_2 (Medical Grade)	African Oxygen (Pty.) Ltd.
Pre-cleaned Slides	"Gold Seal", Clay Adams Inc., N.Y.
Coverslips	Corning No. 1 24 x 40 mm; Corning Glass Works, N.Y.
Kimwipes Type 900-S (N.B. Lint-free)	Kimberley-Clark Corp., Neenah, Wisconsin (not available from local S.A. subsidiary).
Trypsin, "Difco" 1:250 (Certified), or Bacto-trypsin Difco (cert.)	Difco Laboratories Inc., Detroit, Mich. (obt. Protea Holdings, Ltd.)
Buffer, pH 6,8 Tablets	E. Gurr (London) or other.
PBS, Tablets	Oxoid Dulbecco A.

APPENDIX II: Preparation Methods

In all preparations the purest water should be used, preferably double-distilled or distilled – de-ionised in areas with very impure water.

Colchicine: Made up to 0,5% solution in pure water. Should be refrigerated if kept as solution for more than a few days.

Colcemid: Made up and sterilised according to manufacturer's directions. 100mg Colcemid is dissolved in 5,3g 95% ethanol, and 10g propylene glycol is added. A phosphate buffer is made up in pure water:

$Na_2HPO_4.7H_2O$ 0,15g
$NaH_2PO_4.H_2O$ 0,133g

This is added to the Colcemid solution and made up to 100 ml with pure water. This stock solution is sterilised at 10 lbs in an autoclave for 30 minutes. For a 0,05% sterile working solution, pure sterile water is used to dilute the stock solution by half. Both solutions should be refrigerated when possible.

1N HCl: Concentrated reagent-grade hydrochloric acid is added to pure water at the rate of 87,3 ml per 1000 ml.

Methanol – Dry-Ice Freeze Bath: Dry-ice may be used if available, broken into chips in a plastic-lined thermos flask or other suitable insulated container. Reagent-grade methanol is slowly added to the container in small amounts until deep enough for slide immersion. The bath temperature is approximately –85°C.

For field use where dry-ice is not available, CO_2 "snow" is prepared, and used as above. A cylinder of medical-grade (pure) carbon dioxide is *inverted* and an open (valveless) pipe attached directly to the cylinder's outlet. A clean cloth bag is secured over the pipe's outlet and CO_2 "snow" collected by opening the cylinder tap fully. I am indebted to Prof. G.N. Louw of Cape Town University's Zoology Department for originally suggesting this ingenious method.

Cleaning Slides and Coverslips: Several chemical cleaning methods exist (see e.g. Darlington and La Cour 1962), but I have used essentially a manual one passed on by Dr. S. Ohno. "Pre-cleaned" slides and coverslips are placed in a detergent solution, and then individually rubbed with a fine scouring powder (I have used "Ajax") followed by thorough rinsing in tap and distilled water. Naturally all contamination by greasy fingers, etc. must be avoided after this. The slides and slips are briefly drained and then dried with lint-free tissue ("Kimwipes" 900-S). They should not

be stored too long after washing, not more than two days under humid tropical conditions. For use, they are simply wiped clean. The cleaning procedure is not necessary for coverslips used in the final mounting step.

Giemsa Stain: The following standard method was originally communicated to me by Dr. S. Ohno.

0,5 g Giemsa powder
33 ml glycerol
33 ml methanol, absolute

Mix Giemsa powder with glycerin and place in an oven at 60°C for two hours. Remove from oven and let stand for two hours at room temperature before adding methanol. Ripen for two weeks, then filter and keep this stock solution in brown, stoppered bottles. This stain keeps for years.

(a) Standard staining: This may be done on a rack as explained in the text, or in a Coplin jar, diluting stock solution 1:9 with distilled water. The pH should be kept near 7,0, or slightly higher (alkaline). Different stain sources, methanol and water quality all may have a large influence on results.

(b) pH 6,8 buffered Giemsa (G-banding): Dilute stock Giemsa 1:9 with pH 6,8 buffer (see below and Appendix I).

(c) Buffered acetone Giemsa (C-banding): Add 3 ml acetone (reagent) to 17 ml of pH 6,8 buffer for a 15% acetone concentration; use this to dilute stock Giemsa as usual 1:9 for a 10% Giemsa staining solution. Drip onto slides on rack, or use jar.

Trypsin Solution: Follow manufacturer's directions. For Difco Bacto-trypsin, 10 ml sterile distilled water is added to a vial of powder, giving a 5% solution. Aliquots of 1 ml are then frozen immediately. The working solution is prepared by thawing the aliquots at room temperature, and adding 24 ml PBS for a 0,2% trypsin concentration.

PBS (Phosphate-buffered saline): Follow manufacturer's directions. For "Oxoid Dulbecco A" tablets, dissolve one tablet in 100 ml distilled water.

pH 6,8 Phosphate buffer: Follow manufacturer's directions. For E. Gurr tablets, use one per litre of distilled water.

Normal saline: Dissolve 9,46g NaCl in 1 litre distilled water (c. 0,9%).

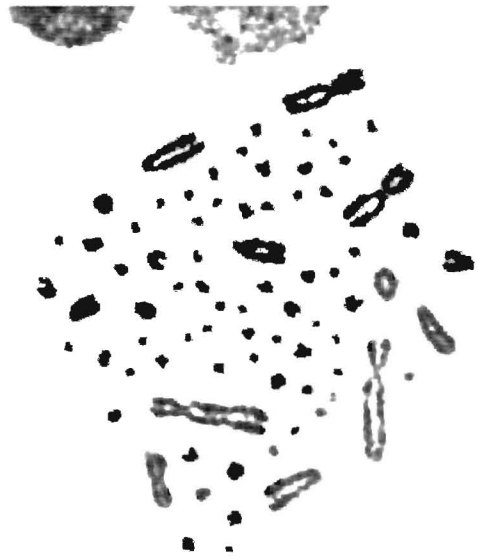
2xSSC (double saline sodium citrate): Dissolve 17,5g sodium chloride and 8,8g Tri-sodium citrate in 1 litre distilled water (0,3 M NaCl and 0,03 M $Na_3C_6H_5O_7$).

$Ba(OH)_2$ (barium hydroxide) solution: Make a saturated solution in distilled water. Allow to clear by precipitation; use only the clear supernatant.

Removal of coverslips and destaining: When "Permounted" preparations must be re-stained, coverslips are removed by soaking in toluene in a well-covered container, coverslip downwards. Hardened mounts may take several days to loosen, but should not be forced in any way: the solvent and gravity must be allowed to complete the job on their own.

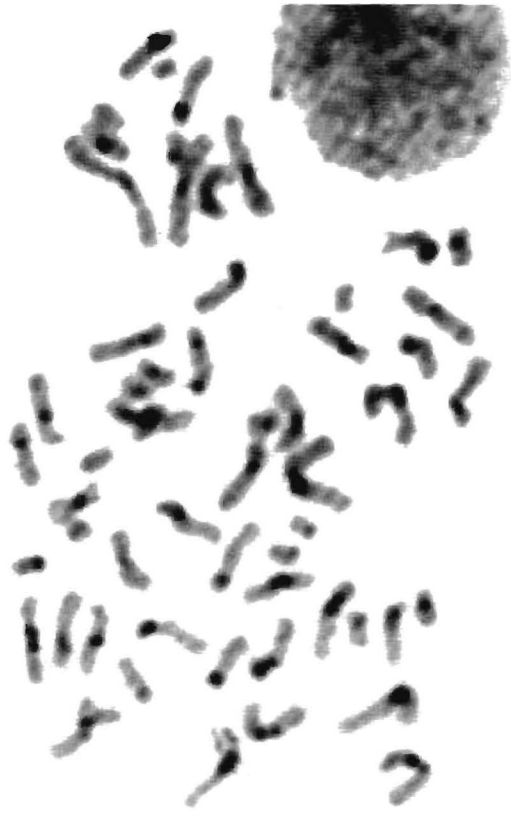
De-staining of Giemsa preparations may be done with a 5 minute alcohol (ethanol or methanol) bath, or by a 30 sec. dip in 50% acetic acid. The latter should not be used on slides intended for banding, however.

PLATE I: Low-power field of view of a good chicken embryo squash preparation, standard Giemsa, with three analysable metaphases. cl. — clumped metaphase (insufficient hypotonic penetration); e — erythrocyte; th — thrombocyte; d — diffuse nuclei representing actively-dividing cell types in interphase and prophase. Chromatin-specific Giemsa does not stain cytoplasm. Approximately x300.



a)

b)



c)

d)

PLATE 2: a) Near-ideal early metaphase from embryonic spleen squash of common quail *Coturnix coturnix* ♀, standard Giemsa. x2060.

b) Prometaphase, slightly clumped, from adult guineafowl *Numida meleagris* ♂, spleen squash, standard Giemsa. This stage is more suitable for high-resolution banding and micro-chromosome analysis than a) x2060.

c) Early metaphase, slightly clumped because of insufficient hypotonic penetration, from spleen of young blue discus-fish *Symphysodon aequifasciata haraldi*. "GTG" method, but bands poorly developed. x3000.

d) Early metaphase (chromatids not separated, probably because of colchicine under-dose) from early embryo of blue discus-fish. "CBG" method showing well-developed C-bands. x3500

Photomicrographs a) and b) on 6,5×9 cm Kodak Contrast Process Ortho sheet film, hard paper. Photomicrographs c) and d) on 35 mm Kodak High Contrast Copy film, hard paper.