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# A Tortoiseshell Male Cat: Chromosome Analysis and Histologic Examination of the Testis

A.S. Pedersen[a] L.C. Berg[a] K. Almstrup[b] P.D. Thomsen[a]

[a]Department of Clinical Veterinary and Animal Sciences, Faculty of Health Sciences, University of Copenhagen, and [b]Department of Growth and Reproduction, Copenhagen University Hospital, Copenhagen, Denmark



**Key Words**

Cat · Infertility · Male cat · Tortoiseshell cat · XXY karyotype

**Abstract**

Tortoiseshell coat color is normally restricted to female cats due to X-linkage of the gene that encodes the orange coat color. Tortoiseshell male cats do, however, occur at a low frequency among tortoiseshell cats because of chromosome aberrations similar to the Klinefelter syndrome in man: the extra X chromosome of a 39,XXY karyotype introduces the possibility of an orange and a non-orange allele which produce the mixture of orange and non-orange coat spotting known as tortoiseshell. We analyzed the chromosome complement of a fibroblast culture and did histological examinations of testicular tissue from a tortoiseshell male cat referred to us. Chromosome analysis using RBA-banding consistently revealed a 39,XXY karyotype. Histological examinations of testis biopsies from this cat showed degeneration of the tubules, hyperplasia of the interstitial tissue, and complete loss of germ cells. Immunostaining using anti-vimentin and anti-VASA (DDX4) showed that only Sertoli cells and no germ cells were observed in the testicular tubules. As no sign of spermatogenesis was detected, we conclude that this is a classic case of a sterile, male tortoiseshell cat with a 39,XXY chromosome complement. 

Tortoiseshell male cats only occur if a male cat has cells with different X chromosomes: one type of X chromosome carrying the orange coat color and another type carrying the non-orange coat color at the same locus. Due to the random inactivation of X chromosomes during embryonic development, this produces patches of orange and non-orange (such as black) coat color called tortoiseshell.

Tortoiseshell male cats can result from several chromosome variations. The majority of these cats have a 39,XXY karyotype that is most likely to occur from meiotic non-disjunction [Hageltorn and Gustavsson, 1981; Leaman et al., 1999; Hartwell et al., 2008; Nicholas, 2010]. Mosaics, such as a 38,XX/39,XXY chromosome constitution, are also frequently observed and are thought to occur due to mitotic non-disjunction. Tortoiseshell male cats can, however, also occur from intrauterine fusion of 2 embryos [Vella et al., 1999]. These cats are designated chimeras and account for the 38,XY/38,XY, 38,XX/38,XY karyotypes described. Cases of 38,XY tortoiseshell male cats are assumed to be either chimeras with an actual 38,XY/38,XY karyotype or a result of somatic reversion [Moran et al., 1984].

Phenotypical characteristics of tortoiseshell male cats vary a lot. Small body composition, female appearance, strong urine smell, infertility, cryptorchidism, and small

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Preben Dybdahl Thomsen
Department of Clinical Veterinary and Animal Sciences
Faculty of Health Sciences, University of Copenhagen
Groennegaardsvej 7, DK–1870 Frederiksberg C (Denmark)
E-Mail pdt@sund.ku.dk

testicular volume have all been reported, but not consistently. Small testicle volume seems to be the most common characteristic affecting these cats, and the testicles often exhibit histopathological changes, such as degeneration of the seminiferous tubules, absence of germinal cells, and hyperplasia of the interstitium [Centerwall and Benirschke, 1975]. Male tortoiseshell cases that have focal areas of normal testicle morphology and active spermatogenesis have been described [Ishihara, 1956; Gregson and Ishmael, 1971; Moran et al., 1984; Kuiper et al., 2003], but these cats all had at least 1 normal 38,XY cell line indicating that a Y chromosome is necessary for maintaining normal morphology and active spermatogenesis.

Here, we report the findings of the chromosome analysis and histological analysis of testicular samples from a 3-year-old tortoiseshell male cat referred to us. It had a 39,XXY karyotype, and the testis had degenerate seminiferous tubules with remnants of Sertoli cells but no spermatogenic cells. The absence of signs of fertility is in agreement with the karyotype of this cat.

## Material and Methods

A 3-year-old tortoiseshell male cat was brought to the animal shelter 'Inge Sørensens Kattehjem', Glostrup, Denmark, for adoption but was later euthanized due to behavioral problems. Fibroblasts from an ear biopsy were cultured, and metaphase spreads were prepared by standard methods. In brief, connective tissue from the inside of the ear was isolated, dissected into 4 × 4 mm pieces, and disintegrated by digestion at 37°C for 3 h in culture medium (DMEM containing GlutaMAX™ 4.5 g/l, 10% fetal calf serum, 1% penicillin/streptomycin, and 1% gentamycin) containing 1.5 g/l collagenase type 1 (Gibco, Life Technologies, Carlsbad, Calif., USA). After sieving through a sterile 70 μm cell filter, the cell suspension was centrifuged for 5 min at 450g, the cells were resuspended and cultured in T75 flasks at 38°C until 50% confluency. Colcemid was added 2 h prior to harvest by trypsination. Hypotonic treatment of cells was done in 0.075 M KCl for 20 min, and the cells were subsequently fixed in fresh 3:1 methanol/acetic acid. To induce RBA-banding, some cultures were incubated with 200 μg/ml 5-bromo-2'-deoxyuridine (BrdU) for 6 h before harvest. Metaphase spreads were prepared by the air-drying technique. Slides used for chromosome counting were Giemsa-stained whereas the RBA-banding used for karyotyping was obtained by staining the BrdU incorporated metaphase spreads with 50 μg/ml acridin orange in 0.07 M Sørensens buffer (pH 6.7) for 20 min. Images of the acridin orange fluorescence were digitally recorded in grey scale using a Leica DMRB microscope equipped with a I3 filter block. The chromosomes were arranged and numbered according to the standard feline karyotype [Ford et al., 1980].

Testicular tissue was fixed in 4% formaldehyde in PBS, imbedded in paraffin, sliced in 5 μm sections, and stained with hematoxylin-eosin and periodic acid Schiff stain for standard histological examination. Sections were scanned on a NanoZoomer (Hamamatsu Photonics, Herrsching, Germany), and NDP.view software (Hamamatsu Photonics) was used for analysis. Immunohistochemistry was performed as described earlier [Rajpert-De Meyts et al., 2003]. A standard indirect peroxidase method was used, and development was done with 3-amino-9-ethylcarbazole (red color). Sections were counterstained with Mayer's hematoxylin. Rabbit anti-DDX4 (Abcam, Cambridge, UK; ab13840) directed against human DDX4 (VASA) was used. For negative controls, serial sections were processed with dilution buffer alone without the primary antibody and did not show any staining (data not shown). Testicular sections from an adult male cat were used as a positive control. For immunofluorescence, the sections were deparaffinized with xylene and rehydrated, followed by damasking of the epitopes for 3 × 5 min with 0.01 M citrate (pH 6) at 1,000 W in a microwave with inverter. After rinsing with PBS, the slides were pre-blocked with 10% goat serum diluted in 0.25% BSA in PBS to avoid unspecific protein binding. After a brief rinse in PBS, the slides were incubated for 1 h with the primary antibody, mouse-anti vimentin (clone V9; DAKO, Glostrup, Denmark) diluted 1:75 with 0.25% BSA in PBS. After rinsing twice in PBS, the slides were incubated for 30 min with the secondary antibody, goat anti-mouse IgG labeled with Alexa 488 (#A11001; Molecular Probes, Life Technologies, Carlsbad, Calif., USA) in a 1:400 dilution with 0.25% BSA in PBS. After rinsing twice in PBS, the slides were stained with 0.1 μg/ml Hoechst (Sigma-Aldrich, St. Louis, Mo., USA) in PBS for 1 min, rinsed 3 times in PBS, and mounted in DAKO fluorescent mounting medium. Images of Hoechst and Alexa 488 fluorescence were captured sequentially in gray scale using the Leica filter block A and I3, respectively, and then superimposed using pseudo-coloring. A negative control was prepared by replacing the monoclonal anti-vimentin antibody with normal mouse IgG. Testicular sections from an adult male cat were used as a positive control.

## Results and Discussion

The 3-year-old tortoiseshell male cat had a male appearance with fat cheeks (fig. 1) and a typical tomcat ruff about the jowls. The body size was normal, but the size of the testes appeared smaller than usual for a 3-year-old cat. A scar from an ovariectomy was observed caudal to the umbilicus, and by consulting the veterinarian performing the surgery previously, it was revealed that the cat had been brought to him as a stray cat and subjected to an ovariectomy under the assumption that it was a female cat. As no ovaries or uterus could be found in the cat's abdomen, the procedure was aborted, and it was assumed that the cat had been through an ovariectomy previously.

Chromosome counting of 160 Giemsa-stained metaphases from fibroblast cultures of an ear biopsy revealed that 148 metaphases (92.5%) carried 39 chromosomes, 5 metaphases (3.1%) showed 38 chromosomes, and 7 metaphases (4.4%) showed less than 38 chromosomes. RBA-banding of 10 randomly selected metaphases consistently revealed a 39,XXY chromosome complement containing

18 homologue chromosome pairs, 2 sex chromosomes (X, Y) and 1 excess X chromosome as illustrated in figure 2. Although we cannot exclude the existence of a rare, second cell line with a 38,XX or 38,XY chromosome constitution, we consider the low frequency of metaphases with 38 chromosomes a preparation artifact induced by the air-drying method, an interpretation which is in agreement with the observation of metaphase spreads with less than 38 chromosomes and with our previous experience with the frequency of chromosome loss by the air drying method (data not shown).

Histological examination of testicular tissue showed degeneration of the seminiferous tubules (fig. 3A, B). The seminiferous tubules appeared to contain few intratubular cells which appeared to be Sertoli cells typically located along the basal membrane. Sertoli cells were, however, found at a much lower frequency than in the normal cat. Pronounced Leydig cell hyperplasia was observed in the interstitium. The testis was apparently without germ cells, and no sign of spermatogenesis was observed. Cellular debris, which was located along the basal membrane, was found sporadically in the tubules. To confirm that the intratubular cells were Sertoli cells and not germ cells, we stained testicular sections for the presence of the well-known germ cell marker VASA (DDX4). We used an antibody directed against human VASA as no other antibody directed against a feline germ cell marker is known. Feline VASA and human VASA, however, share 93% sequence similarity. The anti-VASA antibody clearly stained germ cells in the normal cat and did not stain any cells in the testis of the 39,XXY cat (fig. 3C, D). Moreover, the presumed Sertoli cell origin of the cellular content of the tubules was investigated by vimentin immunofluorescence, as vimentin filaments occur in the cytoplasm of Sertoli cells [Kierszenbaum et al., 1986]. A positive staining of the cytoplasm of the cells on the basal membrane was observed (fig. 3C). Control sections incubated with mouse IgG instead of the anti-vimentin antibody revealed no staining (data not shown), whereas the Sertoli cells in the testicular tubules were consistently highly stained in the adult normal male cat (fig. 3D). Thus, we conclude that the vimentin antibody produces a specific staining of feline vimentin, the VASA antibody of feline germ cells, and that the cellular content of the testicular tubules in the 39,XXY case are Sertoli cells.

Overall it can be stated that the histopathological changes found in the testes of this 39,XXY cat equal those found in other 39,XXY cats. An absence of spermatogenic cells has been indirectly supported by showing that the few remaining cells in the testicular tubules are most like-

**Fig. 1.** Male phenotype of the tortoiseshell male cat. Note the characteristic cheeks of normal male cats (courtesy of F. Andersen, Inge Sørensens Kattehjem, Glostrup, Denmark).

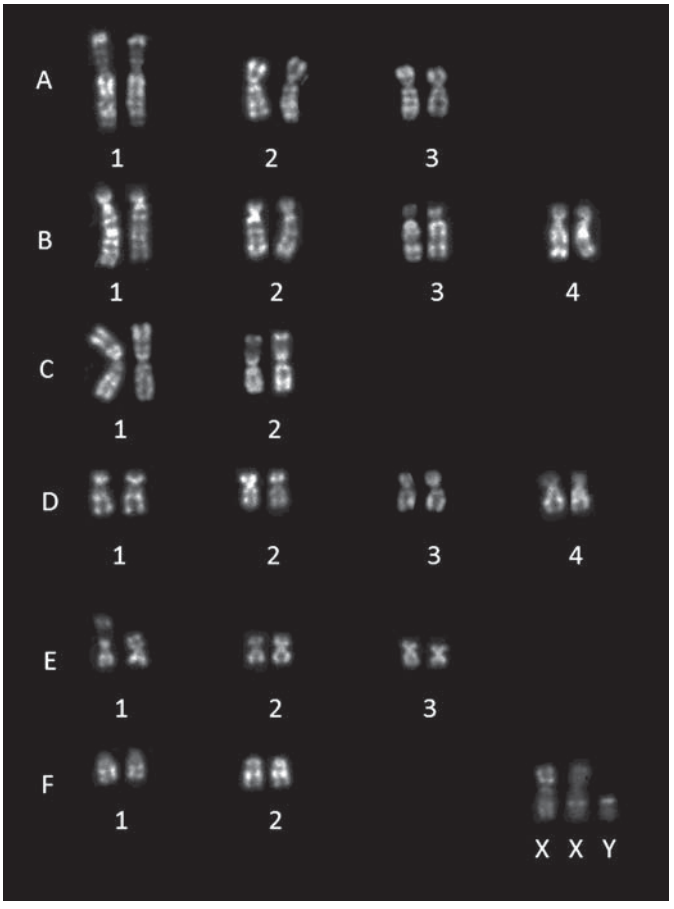


**Fig. 2.** RBA-banded karyotype from the tortoiseshell male cat arranged and numbered according to Ford et al. [1980]. The karyotype shows a 39,XXY trisomy.

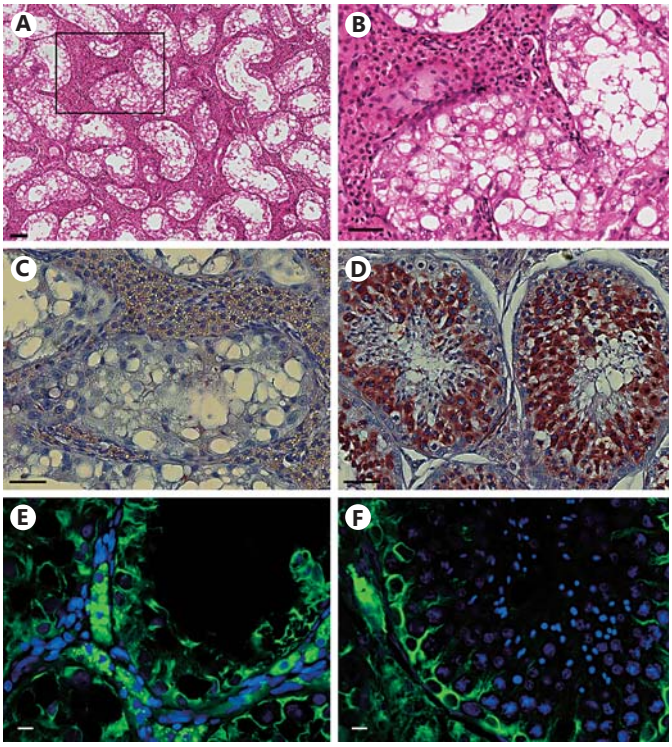


**Fig. 3.** Histological examination and immunofluorescence staining of the testis from a tortoiseshell male cat and from a normal male cat. **A** Hematoxylin-eosin staining shows degeneration of the seminiferous tubules and hyperplasia of the interstitial tissue. Bar = 100 μm. The **insert** shows the part enlarged in **B**. **B** Degenerated tubule with few Sertoli cells. No germ cells are observed, and Leydig cell hyperplasia is evident. Bar = 50 μm. **C** Immunohistochemistry on a testicular section from the 39,XXY cat with the antibody directed against human VASA. No staining is observed in the 39,XXY cat, but intense staining in germ cells is observed in **D** when sections of a normal adult cat are investigated. Bars = 50 μm. **E** Immunofluorescence staining of vimentin (green) in tubular sections from the 39,XXY tortoiseshell male cat. Labeling is observed on cells located on the basal membrane. Bar = 10 μm. **F** Immunofluorescence staining of vimentin in testicular sections from a normal adult male cat. Green labeling appears in the cytoplasm of Sertoli cells located at the basement membrane. Hoechst staining (blue) marks nuclei. Bar = 10 μm.

ly Sertoli cells. Thus, we consider this cat a classic XXY case which in many aspects shows a similar testicular pathology (and to some degree phenotype) as men with 47,XXY Klinefelter syndrome [Aksglaede et al., 2011]. Germ cells can be found in prepubertal boys with Klinefelter syndrome, but at onset of puberty the testes are degenerated and usually only Sertoli cells are found inside the seminiferous tubules in an adult Klinefelter man [Aksglaede et al., 2011]. The cat was detected primarily because it had a tortoiseshell coat color pattern, and it would be very interesting to investigate whether germ cell are present in the prepubertal testis of a 39,XXY cat. This case history also illustrates the risk in assuming a tortoiseshell cat to have been spayed if no ovaries or uterus can be found at ovariectomy.

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## References

Aksglaede L, Skakkebaek NE, Almstrup K, Juul A: Clinical and biological parameters in 166 boys, adolescents and adults with nonmosaic Klinefelter syndrome: a Copenhagen experience. Acta Paediatr 100:793–806 (2011).

Centerwall WR, Benirschke K: An animal model for the XXY Klinefelter's syndrome in man: tortoiseshell and calico male cats. Am J Vet Res 36:1275–1280 (1975).

Ford CE, Pollock DL, Gustavsson I: Proceedings of the First International Conference for the Standardisation of Banded Karyotypes of Domestic Animals. University of Reading, Reading, England. 2nd–6th August 1976. Hereditas 92:145–162 (1980).

Gregson NM, Ishmael J: Diploid-triploid chimerism in 3 tortoiseshell cats. Res Vet Sci 12: 275–279 (1971).

Hageltorn M, Gustavsson I: XXY-trisomy identified by banding techniques in a male tortoiseshell cat. J Hered 72:132–134 (1981).

Hartwell LH, Hood L, Goldberg ML, Reynolds AE, Silver LM, Veres RC: Genetics from Gene to Genome, ed 3, (McGraw Hill, New York 2008)

Ishihara T: Cytological studies on tortoiseshell male cats. Cytologia 21:391–398 (1956).

Kierszenbaum AL, Crowell JA, Shabanowitz RB, Smith EP, Spruill WA, Tres LL: A monoclonal antibody recognizes a form of intermediate filament protein in rat Sertoli cells that is not present in seminiferous peritubular cells. Biol Reprod 35:227–238 (1986).
Kuiper H, Hewicker-Trautwein M, Distl O: Zytogenetische und histologische Untersuchungen an vier Schildpattkatern. Dtsch Tierarztl Wochenschr 110:457–461 (2003).
Leaman T, Rowland R, Long SE: Male tortoiseshell cats in the United Kingdom. Vet Rec 144:9–12 (1999).
Moran C, Gillies CB, Nicholas FW: Fertile male tortoiseshell cats mosaicism due to gene instability? J Hered 75:397–402 (1984).
Nicholas FW: Introduction to Veterinary Genetics, ed 3, (Wiley-Blackwell, Chichester 2010).
Rajpert-De Meyts E, Bartkova J, Samson M, Hoei-Hansen C, Frydelund-Larsen L, et al: The emerging phenotype of the testicular carcinoma in situ germ cell. APMIS 111:267–278; discussion 278–279 (2003).
Vella CM, Shelton LM, McGonagle JJ, Stanglein WT: Robinson's Genetics for Cat Breeders and Veterinarians, ed 4, (Butterworth-Heinemann, Burlington 1999).