THE EFFECT OF FLUORIDE ON THE PHYSIOLOGY OF THE PINEAL GLAND

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ABSTRACT

The purpose was to discover whether fluoride (F) accumulates in the pineal gland and thereby affects pineal physiology during early development. The [F] of 11 aged human pineals and corresponding muscle were determined using the F-electrode following HMDS/acid diffusion. The mean [F] of pineal was significantly higher ($p < 0.001$) than muscle: 296 ± 257 *vs.* 0.5 ± 0.4 mg/kg respectively. Secondly, a controlled longitudinal experimental study was carried out to discover whether F affects the biosynthesis of melatonin, (MT), during pubertal development using the excretion rate of urinary 6-sulphatoxymelatonin, (aMT6s), as the index of pineal MT synthesis. Urine was collected at 3-hourly intervals over 48 hours from two groups of gerbils, *(Meriones unguiculatus*), low-F (LF) and high-F (HF) (12 f, 12 m/group): under LD: 12 12, from prepubescence to reproductive maturity (at 9-12 weeks) to adulthood, i.e., at 7, 9, 11½ and 16 weeks. The HF pups received 2.3 µg F/g BW/day from birth until 24 days whereafter HF and LF groups received food containing 37 and 7 mg F/kg respectively and distilled water. Urinary aMT6s levels were measured by radioimmunoassay. The HF group excreted significantly less aMT6s than the LF group until the age of sexual maturation. At 11½ weeks, the circadian profile of aMT6s by the HF males was significantly diminished but, by 16 weeks, was equivalent to the LF males. In conclusion, F inhibits pineal MT synthesis in gerbils up until the time of sexual maturation. Finally, F was associated with a significant acceleration of pubertal development in female gerbils using body weights, age of vaginal opening and accelerated development of the ventral gland. At 16 weeks, the mean testes weight of HF males was significantly less ($p < 0.002$) than that of the LF males. The results suggest that F is associated with low circulating levels of MT and this leads to an accelerated sexual maturation in female gerbils. The results strengthen the hypothesis that the pineal has a role in pubertal development.

DECLARATION

I, Jennifer Luke, hereby declare that this research paper is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of Surrey, Guildford. It has not been presented for any degree at any other University.

J Luke

29th day of March 1997

In memory of Lynne
1943 - 1984

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LIST OF ABREVIATIONS

5HT	serotonin
aMT6s	6-sulphatoxymelatonin
ANOVA	analysis of variance
AUC	area under the curve
B/B_0	a common response variable in RIA systems
b.pt.	boiling point
BW	body weight
CCT	cranial computed tomogram
CNS	central nervous system
CSF	cerebrospinal fluid
CSU	charcoal stripped urine
CV	coefficient of variation
cpm	counts per minute
DCC	dextran-coated charcoal
d-H_2O	double-distilled water
F	fluoride ion
[F]	fluoride concentration
GCMS	gas chromatography-mass spectrometry
h	hour
HA	hydroxyapatite
HF	high-fluoride group
HIOMT	hydroxyindole-*O*-methyltransferase
HMDS	hexamethyldisiloxane
L	litre
LD: 12 12	12 hour light, 12 hour dark cycle
LF	low-fluoride group
LFNM	low-fluoride non-monitored group
MT	melatonin
MW	molecular weight
NAS	*N*-acetylserotonin
NAT	*N*-acetyltransferase
NSBs	non-specific binding tubes
OH	hydroxyl ion
PC	pineal calcification
ppb	part per billion
QCs	quality control tubes
RIA	radioimmunoassay
rpm	revolutions per minute
sc	subcutaneous
SCG	superior cervical ganglion
SCGX	superior cervical ganglionectomy
SCN	suprachiasmatic nuclei
SD	standard deviation
SEM	standard error of the mean
SNK	Student-Newman-Keuls test
T/P ratio	tissue water to plasma water concentration ratio
vs.	versus

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Chapter 1 - Background Information

1.1 Introduction

In this study I attempted to discover whether fluoride (F) has pathophysiological effects on the pineal gland: a feasible proposition if F accumulates in the pineal and can thereby influence its physiology. The pineal gland, or seat of the soul as it is colloquially called, is situated near the anatomical centre of the brain. It is an integral part of the central nervous system (CNS). Fluoride metabolism in the CNS has not been systematically studied. It is generally believed that F has no effect on the CNS because it is excluded from brain by the blood-brain barrier (Whitford *et al*, 1979). Whole brain has a low F-content like normal soft tissues elsewhere in the body.

It is remarkable that the pineal gland has never been analysed separately for F because it has several features which suggest that it could accumulate F. It has the highest calcium concentration of any normal soft tissue in the body because it calcifies physiologically in the form of hydroxyapatite (HA). It has a high metabolic activity coupled with a very profuse blood supply: two factors favouring the deposition of F in mineralizing tissues. The fact that the pineal is outside the blood-brain barrier suggests that pineal HA could sequester F from the bloodstream if it has the same strong affinity for F as HA in the other mineralizing tissues.

The intensity of the toxic effects of most drugs depends upon their concentration at the site of action. The mineralizing tissues (bone and teeth) accumulate high concentrations of F and are the first to show toxic reactions to F. Hence, their reactions to F have been especially well studied. If F accumulates in the pineal gland, then this points to a gap in our knowledge about whether or not F affects pineal physiology. It was the lack of knowledge in this area that prompted my study.

Children are now exposed to more F than ever before. Fluorides are the cornerstone of all caries preventative programs. The substantial reduction in the incidence of dental caries in the western world over the past fifty years has been largely attributed to the access to fluoridated water supplies and the increased exposure to F in dental products. The fluoridation of water supplies is an important public health measure. It is endorsed by the WHO, the European Union directives, the Royal College of Physicians, the Royal College of General Practitioners, the BMA, and the medical and dental professions (Samuels, 1993).

Despite the endorsements, the prophylactic use of F in dentistry has been a controversial subject for decades. One recent study reported an increase in osteosarcomas in male F344/N rats which had received drinking water containing 100-175 mg NaF/L (45-79 mg F/L) for two years (NTP, 1990). Following this report, three critical bodies analysed the public health benefits and risks from chronic F-exposure by reviewing the evidence from human epidemiological studies of the relationship between cancer and water fluoridation and also carcinogenicity studies in rodents. They unanimously

agreed that F is safe and effective if used appropriately. The use of F is not associated with an increased cancer risk in humans. Dental fluorosis is the only adverse effect associated with the chronic ingestion of relatively low F-levels (Kaminsky *et al*, 1990; USPHS, 1991; NRC, 1993). Further research is required on the effects of F on the reproductive system in animals and humans (USPHS, 1991).

Dental fluorosis (defective, hypomineralized enamel) occurs when excessive amounts of F reach the growing tooth during its developmental stages. The manifestations of fluorosis range from barely noticeable opacities to severely pitted teeth. The greater the F-exposure during tooth formation the greater is the likelihood of dental fluorosis developing and the more severe is the pathology. The F-concentration at which fluorosis becomes apparent in a population corresponds to a daily intake of about 0.1 mg F/kg body weight (BW) up to the age of 12 years although there is no firm consensus on this issue. In fact, a high prevalence and severity of dental fluorosis was reported in populations with an estimated daily F-intake of less than 0.03 mg F/kg BW (Bælum *et al*, 1987).

The so-called 'optimal' concentration of F in community water is defined as the concentration of F which gives maximum caries reduction and causes minimum dental fluorosis, i.e., between 0.7 and 1.2 mg/L depending on the mean ambient temperature. At the time of Dean's original studies, there was a 10-12 percent prevalence of mild dental fluorosis in children in the 1 mg F/L areas (Dean *et al*, 1941, 1942). This was 'accepted' in return for the benefits in caries reduction: a classic public health trade-off. In the 1930s and 1940s, virtually the only source of F was in the drinking

water. Today, F is ubiquitous in the environment which means that man's daily F-intake comes from several sources besides tap water.

Systemic F-exposure to children has increased (Leverett, 1991). Mild dental fluorosis is now more common than one would predict on the basis of Dean's findings in the late 1930s and early 1940s: in fluoridated and non-fluoridated communities (Leverett, 1986; Pendrys and Stamm, 1990; USPHS, 1991). Several recent studies report prevalence rates in the 20 and 80 percent range in areas with fluoridated water (Levy, 1994). The prevalence of 0.9 percent (recorded in the pre-fluoride days) in areas containing less than 0.4 mg F/L in the water has increased to 6.6 percent (USPHS, 1991). The prevalence of moderate to severe dental fluorosis has increased (Williams and Zwemer, 1990; USPHS, 1991; Lalumandier and Rozier, 1995). The increased prevalence of dental fluorosis is causing concern within the scientific community because it is an early sign of F-toxicity and evidence that some children are now getting more F than is good for them. The issue has the potential to become a significant dental health problem.

Of all the tissues, the developing enamel organ is assumed to be most sensitive to the toxic effects of F. It contains significantly higher concentrations of F and calcium than other soft tissues. The enamel organs of 9-day-old rats contained significantly higher F-levels than corresponding soft tissue (0.14 *vs.* 0.015 mg/kg). Following oral administration of F to the rat pups (0.5 mg/kg BW), the [F] of the enamel organ reached peak values (0.19 mg/kg) in 30 minutes. The enamel organ may be relatively sensitive to increased systemic F-intake because it accumulates F (Bawden *et al*, 1992).

Although the exact mechanism responsible for enamel fluorosis is not known, F may have specific effects on the normal activity of ameloblasts, developing enamel matrix and proteolytic activity in the maturing enamel (DenBesten and Thariani, 1992). The transition/early-maturation stage of amelogenesis is most susceptible to the effects of an increased plasma F-concentration. The aesthetically important maxillary central incisors are most vulnerable to F at 22-26 months (Evans and Stamm, 1991).

Alongside the calcification in the developing enamel organ, calcification is also occurring in the child's pineal. It is a normal physiological process. A complex series of enzymatic reactions within the pinealocytes converts the essential amino acid, tryptophan, to a whole family of indoles. The main pineal hormone is melatonin (MT). For some reason, young children have the highest levels of plasma MT. They also have higher plasma F-levels (recommended from a dental perspective) than they did 50 years ago. An increasing number of children suffer from mild dental fluorosis: evidence that they received too much F during the first few years of life. If F accumulates in the pineal gland during early childhood, it could affect pineal indole metabolism. In much the same way that high local concentrations of F in enamel organ and bone affect the metabolism of ameloblasts and osteoblasts.

If F influences the high pineal MT output during early development, then the functions of the pineal may also be compromised (given that MT is the main mediator of pineal function). One putative function of the pineal is its involvement in the onset of puberty. If F compromises pineal function by altering

the high rate of synthesis of MT during childhood, does this manifest as an alteration in the timing of puberty?

Although the extrapolation of results from animal studies to the human situation is difficult, this project may identify a potential health risk to humans. Therefore, the results will either affirm the safety of the extensive use of F in dentistry or suggest that harmful effects on human health have already occurred: either way, this investigation is worthwhile.

1.2 Review of the Literature

To the best of my knowledge, the Newburgh-Kingston study is the only reference on the effect of F on the timing of puberty in humans. It is the largest, most ambitious paediatric survey carried out to demonstrate the safety of water fluoridation. The New York State Department of Health initiated the study in 1944 because they realized that there would ultimately be a need for a long-term evaluation of any possible systemic effects as well as the dental changes from drinking fluoridated water over a long period of time.

Similar groups of children were selected for long-term observation from Newburgh (fluoridated to 1.0 to 1.2 mg/L in 1945) and Kingston (essentially F-free for the duration of the study). Newburgh and Kingston were chosen because they were well-matched: both were situated on the Hudson River about 35 miles apart with similar upland reservoir water supplies; both had populations of about 30,000 with similar demographic characteristics, social and economic conditions, levels of dental

care, etc. In Newburgh, out of 817 children (aged from birth to nine years) who were selected in 1945, 500 were examined in 1954-1955; in Kingston, out of 711 children who were selected in 1945, 405 were examined in 1954-1955.

The medical and dental examinations began in 1944, and were repeated periodically until 1955. An assessment of any possible systemic effects arising from the consumption of fluoridated water was made by comparing the growth, development and the prevalence of specific conditions in the two groups of children as disclosed by their medical histories, physical examinations, and laboratory and radiological evidence. The age of onset of menstruation in girls was used as an index of the rate of sexual maturation.

At the end of ten years, the investigators reported no adverse systemic effects from drinking fluoridated water because no significant differences were found between the results from the two groups. The average age of first menarche was earlier among girls in Newburgh than those in Kingston: 12 years *vs*. 12 years and 5 months respectively (Schlesinger *et al*, 1956). Although this difference was not considered important, it does suggest an association between the use of fluoridated drinking water and an earlier onset of sexual maturation in girls. The Newburgh girls had not had a lifelong use of fluoridated water. For the first two years or so, they received unfluoridated water. Furthermore, their only source of F was from the drinking water.

1.3 Sources of Fluoride

1.3.1 Food

The normal daily F-intake is negligible (less than 0.01 mg) during the first few months of human life, because human breast milk contains merely a trace of F (6 to 12 ng/ml): regardless of the F-intake to the nursing mother. Ekstrand and co-workers (1981) analysed plasma and milk samples from five nursing mothers after they had taken an oral dose of 1.5 mg F. There was an immediate ten-fold increase in the [F] of plasma (70-86 ng/ml) within 30 minutes of dosing but the [F] of breast milk remained constant throughout the day (2-8 ng/ml). The mean F-concentrations of human breast milk were 8.9 and 5.0 ng/ml from nursing mothers living in 1.7 and 0.2 mg F/L areas respectively (Esala *et al*, 1982); 6.8 ± 0.4 and 5.3 ± 0.4 ng/ml (± SEM) from nursing mothers living in 1.0 and 0.2 mg F/L areas respectively (Spak *et al*, 1983).

The reason for the limited transfer of F from plasma to breast milk is unknown. It has been suggested that the physiological plasma-milk barrier actively protects the newborn from the toxic effects of F (Ekstrand *et al*, 1981). Cow's milk, like human milk, contains low levels of F (0.017 mg/L) even when F is added to the cow's food or drinking water (McClure, 1949). Breast-fed infants (or infants bottle-fed with cow's milk) are in negative F-balance: more F is excreted in the urine than is ingested in the diet. During the period of breast feeding, F (deposited in foetal bone during pregnancy) is mobilized and

released into the extracellular fluids and subsequently excreted into urine. Therefore, early human development has always occurred in a virtually F-free milieu even in the high-F areas: a phenomenon which lasts until the age of weaning and the introduction of solid foods.

In contrast, the F-intake to bottle-fed infants living in fluoridated areas depends upon the [F] of: a) the water used to reconstitute the feed; b) the powdered formula-feed itself. Bottle-fed infants in fluoridated areas can receive 1.1 mg F from day 1: 150-200 times more F per day than breast-fed infants, i.e., 1100 *vs*. 5-10 µg/day (Ekstrand, 1989). The normal pharmacokinetics of F during infancy is reversed. Bottle-fed infants in fluoridated areas retain more than 50% of the ingested F-dose in the mineralizing tissues (Ekstrand *et al*, 1984; 1994).

Man's daily intake of F from food is low. Fresh, unprepared vegetables, fruits, pulses, roots, nuts, etc., rarely contain more than 0.2 to 0.3 mg F/kg (WHO, 1984). Most plant species have a limited capacity to absorb F from the soil even when F-containing fertilisers are applied (Davison, 1984). The flesh of meat, poultry and fish, (free from bone), contains low levels of F because virtually all F in animals occurs in their bones and teeth. The skin and bones of tinned salmon and sardines contain 8 and 500 mg F/kg respectively because the fish are exposed to relatively high levels of F (1.2-1.4 mg/L) in sea-water (Jenkins, 1990).

Nevertheless, most of man's daily intake of F is derived from food unless he drinks fluoridated water (or water containing naturally high levels of F) or is exposed to F from industrial contamination (Underwood, 1977). Studies have shown that adults living in non-fluoridated areas receive low levels of F from food alone: 0.16 mg F/day (Machle *et al*, 1942); 0.2 to 0.3 mg F/day (McClure, 1949); 15- to 19-year-old American males receive 0.27 ± 0.03 mg F/day (Singer *et al*, 1985). Six-month-old infants and two-year-old children receive less than 0.2 mg F/day from food (Ophaug *et al*, 1980a; 1980b).

The dispersion of F into food was an unplanned consequence of water fluoridation. Processed food and beverages contain significant amounts of F if they are manufactured in towns supplied with fluoridated water. Such foodstuffs can be distributed and consumed in non-fluoridated communities. Their consumption produces an average F-intake of 1.0 to 2.0 mg/day (Marier and Rose, 1966).

1.3.2 Drinking Water

Man has evolved over the millenia drinking water with low F-levels, e.g., surface waters, collected rain or melted snow. Today, most large communities obtain their drinking water from supplies of fresh surface waters which contain between 0.01 to 0.3 mg F/L (WHO, 1984). In the 1940s, 95.7% of the population of USA received water containing less than 0.5 mg F/L (Hill *et al*, 1949); in the 1980s, most water supplies in the USA contained less than 0.3 mg F/L unless they had been

artificially fluoridated (Centers for Disease Control, 1985); in Norway, 96% of the population consumed water containing less than 0.25 mg F/L (Lökken and Birkeland, 1978).

In some parts of the world, particularly rural areas, populations use water from deep wells or bores. Underground water has a greater opportunity to contact fluoriferous material. Ground-waters contain varying amounts of F: less than 0.1 mg/L to more than 25 mg/L (USPHS, 1991). Bottled spring waters (still) contain less than 0.1 mg F/L. The drinking water from wells in districts with endemic fluorosis in USA contained 2.0 to 16.8 mg F/L (McKay, 1933).

1.3.3 Dental Products

Since 1945, there has been a steady increase in the use of F in various forms and concentrations for the prevention of dental caries. The self-applied topical fluorides include: fluoridated-toothpaste (1000-1500 mg F/kg) which typically provides 0.5-1.5 mg F per brushing when 0.5-1.5 g toothpaste (1000 mg F/kg) is dispensed; cosmetic mouth-wash sold over the counter (230 mg F/L) provides 1.1-3.4 mg F when 5-15 ml is used; high-F mouth-rinses by prescription (900 mg F/L) (Shulman *et al*, 1995). The professionally-applied topical fluorides include: F-gels (12,300 mg F/kg); F-varnishes. The F-preparations intended for systemic use include F-tablets and F-drops.

Young children swallow substantial amounts of toothpaste or mouth-rinse because they have poor control over the

swallowing reflex. The F in toothpaste and mouth-rinse is readily bioavailable (Ekstrand and Ehrnebo, 1980) and virtually all the ingested F is absorbed in the gastrointestinal tract. A two-year-old child swallows about 60% and a five-year-old about 34% of the F in toothpastes (Simard *et al*, 1989, 1991). In real terms, 2-5-year-old children ingest 0.5 to 0.75 mg F per use (Murray, 1986); 7-13-year-old children ingest 0.4 to 1.2 mg F per use (Bell *et al*, 1985). Therefore, some children exceed their optimal daily F-allowance from toothpaste alone. Consequently, there is an increase in plasma F-levels.

1.4 Fluoride in Plasma

In low-F areas, the mean [F] in plasma from fasting, middle-aged adults is about 0.01 mg/L ranging from 0.004 to 0.02 mg/L or 0.5 μM (Murray *et al*, 1991). In fluoridated areas, the plasma F-levels range from 0.02 to 0.04 mg/L, about 1 μM (Ekstrand *et al*, 1988).

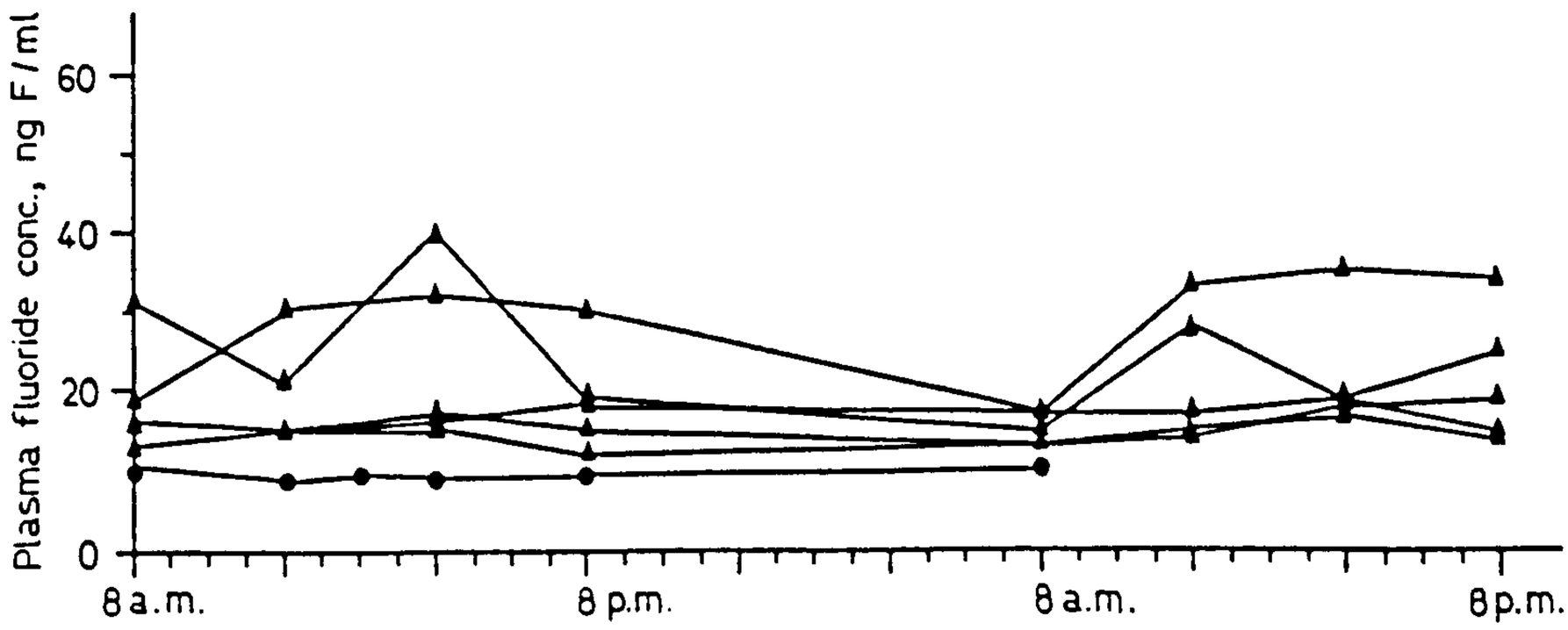

Fig 1.1 Plasma F-concentrations in five subjects living in a 1.2 mg F/L area since birth (▲) and the mean plasma F-concentration from five subjects living in a 0.25 mg F/L area since birth (●). Reproduced from Ekstrand, 1978, with permission.

Figure 1.1 presents the plasma [F] curves from five subjects from areas with 1.2 and 0.25 mg F/L in the water supply. To simplify the graph, the mean plasma [F] from the five subjects from the 0.25 mg F/L area is presented because their plasma [F] were similar throughout the 24-h period (9.4 ± 2.2 ng/ml, ± SD). The plasma [F] in subjects from the fluoridated area were higher than those in the low-F subjects throughout the day and two subjects in the high-F area showed signs of a diurnal variation (Ekstrand, 1978).

Less is known about the [F] in plasma from infants and young children. The plasma [F] in the suckling rat is very low: about 0.002 mg/L (Bawden *et al*, 1986; 1992) which is probably due to the low [F] of rat milk (less than 0.01 mg/L) (Drinkard *et al*, 1985). Presumably the plasma [F] in infants are also very low: only increasing when F is administered. Fluoride is rapidly cleared from the bloodstream in infants and young children by sequestration by the rapidly mineralizing tissues.

Figure 1.2 presents the 24-h plasma [F] in a male subject in four separate experiments in which he was given four different single oral doses of F (Ekstrand *et al*, 1977). It can be seen that F is rapidly and readily absorbed from the gastrointestinal tract. The F-levels in plasma start to rise within a few minutes after F-intake and take 30 minutes to reach the peak values and several hours to return to basal levels.

Plasma F-levels are not homeostatically regulated in the classical sense. Peak values are proportional to the amount of F ingested and inversely proportional to body weight (Ekstrand *et al*, 1988).

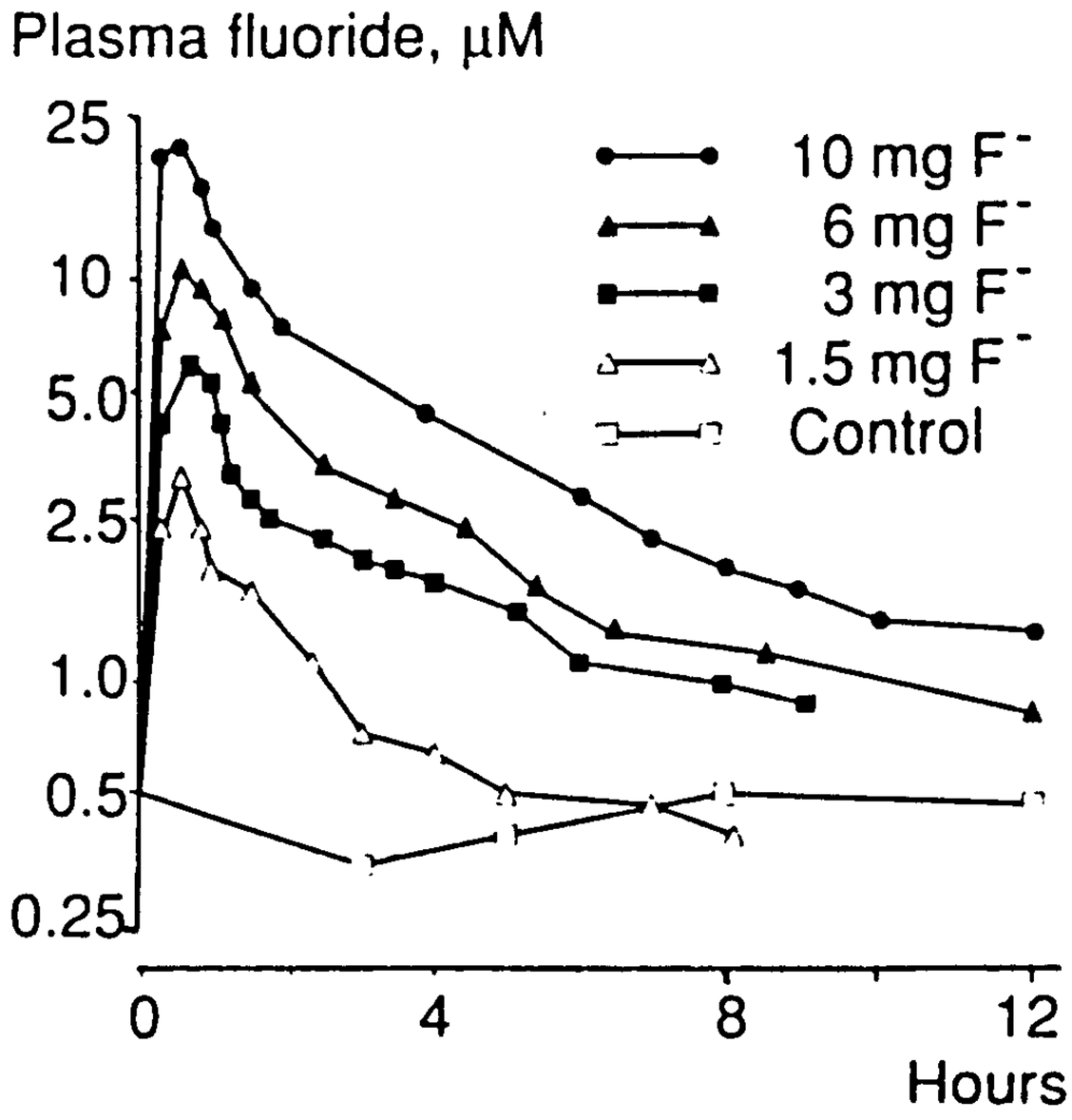

Fig. 1.2 Plasma F-concentrations in a healthy male aged 23 years after an intake of four different oral doses of F. Reproduced from Ekstrand *et al*, 1977, with permission.

Young children regularly receive repeated doses of F from swallowing fluoridated toothpaste. When 3- to 4-year-old children ingested 0.6 g toothpaste (1000 mg F/kg), the mean peak plasma F-concentration (4 µM) was almost the same as when they received 0.5 mg NaF tablet (Ekstrand *et al*, 1983). One hour after the routine application of topical F-gels to the teeth, children had peak plasma F-levels ranging from 16 to 76 µM. The highest value (76 µM F) occurred in a 5-year-old child. The plasma F-levels remained high for two hours (Ekstrand *et al*, 1981).

1.5 Fluoride in Calcified Tissues

Approximately 99% of all the F in the human body is found associated with calcified tissues because F is avidly attracted to hydroxyapatite (HA). The rapid uptake of F by the mineralized tissues is one reason for the low levels of F in plasma (Jenkins,

1990). The rate of clearance of F from plasma exceeds that for calcium (Costeas *et al*, 1971). Whole enamel contains about 100 mg F/kg although the outer 2 μm of surface enamel from permanent teeth contains high levels of F ~ 1000-3000 mg/kg depending upon the [F] of the drinking water (Murray *et al*, 1991). Dentine and cementum contain about 300 and 1000 mg F/kg respectively (Newbrun, 1986).

Bone is the main store of F and its [F] is a good indicator of previous F-exposure. Bone F-content directly correlates with F-intake (Turner *et al*, 1992); with age of the subject, and the duration of exposure (Kuo and Stamm, 1974; Parkins *et al*, 1974; Charen *et al*, 1979). Fluoride is not evenly distributed throughout bone. The [F] of trabecular bone is higher than cortical bone. The [F] of endosteal and periosteal surfaces are higher than the central parts of bone. The deposition of F also depends upon the stage of development, rate of growth and the vascularity of the bone; the surface area and size of the crystallites. Therefore, when bone samples are taken for F-analysis, the sites must be standardized (Jenkins, 1990).

Under normal circumstances, young or middle-aged adults retain about one half of the absorbed F in the calcified tissues and excrete the other half in the urine. Infants and young children retain more F than adults because they have a greater proportion of their skeletons available to the circulation and are actively laying down bone mineral (Whitford, 1996). Infants and young children excrete less of the daily F-intake in the urine.

Several studies have determined the [F] of human bone. The mean [F] of bone ash from elderly subjects was: 2085 ± 1113 mg/kg (Charen *et al*, 1979); 1834 mg/kg and the highest value was in a 79-year-old subject: 3708 mg F/kg (Ebie *et al*, 1992). Jenkins (1990) reported F-levels as high as 6000 mg/kg in bone ash from elderly subjects living in Hartlepool where water supplies contained 2 mg F/L for most of the past 100 years .

Table 1.1 presents the F-concentrations of ashed bone in previous laboratory studies in which the animals were given different daily doses of F in either the drinking water or the food.

Table 1.1 Fluoride content of bone ash in previous animal studies

Species	water mg F/L	food mg F/kg	Duration	bone [F] mg/kg mean	Reference
rat	15	<1.2	12 w	2223	Turner *et al*, 1995
rat	15	<1.2	24 w	2344	Turner *et al*, 1995
rat	50	<1.2	12 w	5764	Turner *et al*, 1995
rat	50	<1.2	24 w	7081	Turner *et al*, 1995
rat	~30	0.76	6 w	2672	Whitford, 1991
rat	75	37	6 w	4063	Ebie *et al*, 1992
rat	45	~ 8	103 w	3648	NTP, 1990
mouse	tap	4 mg NaF/kg BW/d	95 w	4405	Maurer *et al*, 1991
rat	tap	4 mg NaF/kg BW/d	95 w	5014	Maurer *et al*, 1991
mouse	tap	10 mg NaF/kg BW/d	95 w	7241	Maurer *et al*, 1991
rat	tap	10 mg NaF/kg BW/d	95 w	8849	Maurer *et al*, 1991

The principal mineral of skeletal tissues is the particular crystallized form of calcium phosphate known as apatite, $Ca_{10}(PO_4)_6X_2$: when X = OH, the mineral is hydroxyapatite (HA); when X = F, the mineral is fluorapatite. Impure HA is the prototype of one of the major constituents of bone and teeth. Several different ions can occupy positions in the apatite crystal with only minor changes in its dimensions and form. Most of the F replaces OH in

the apatite crystal lattice because F and OH have almost the same size and charge. Fluorapatite (38,000 mg F/kg) is formed when all the OH ions are replaced by F. A fraction of the F replaces carbonate or bicarbonate groups situated within or at the surface of the crystallites.

1.6 Fluoride in Soft Tissues

Normal soft tissues contain negligible amounts of F irrespective of the F-intake or age: less than 1 mg/kg, (WHO, 1984). Of the soft tissues, the kidney has the highest [F]: 0.7 mg F/kg (fresh) (Gettler and Ellerbrook, 1939); 3.4 mg F/kg (ashed) (Mohamedally, 1984). This is due to retained urine in the renal tubules and collecting ducts (urine has a higher [F] than plasma) (Whitford and Taves, 1973). Soft tissues can accumulate F if they contain ectopic calcification, e.g., the placenta near the end of pregnancy, atheromatous plaques in the aortic lining.

Studies using radiofluoride (^{18}F) have demonstrated that administered F is rapidly distributed throughout the body compartments and reaches an equilibrium with F in plasma. Intracellular [F] are 10-50% lower than those of plasma but the ratio of the concentrations is constant. The blood flow and the extent of the capillary bed are important in the distribution kinetics of F since equilibrium between [F] in intra- and extracellular fluids is established more rapidly in well-perfused tissues than in less vascular tissues. Fluoride readily penetrates intracellular spaces as judged by the isotope-levels in tissue-water-to-plasma-water concentration ratios (T/P ratios). The ratios for most soft tissues

range between 0.4 and 0.9 which means that soft tissues do not bind F. Brain has the lowest T/P ratio, i.e., 0.08. The blood-brain barrier is relatively impermeable to F in short-term studies (Whitford *et al*, 1979).

1.7 Blood-Brain Barrier

Studies have shown that the human brain contains low levels of F: 0.53 mg/kg (wet weight) (Gettler and Ellerbrook, 1939); 1.8 mg/kg (wet weight) after exposure to high atmospheric [F] (Call *et al*, 1965); 0.9 mg/kg from a person exposed to a large unknown amount of F and whose plasma [F] was 4.9 mg/L (Singer and Ophaug, 1982). Using neutron activation analysis, the highest F-levels were found in mid-brain, pons and medulla in the rat brain (Chan *et al*, 1983). Several regions of rat brain accumulate significant amounts of F following the rat's intake of drinking water with 125 mg F/L for 20 weeks (starting at weaning) (Mullenix *et al*, 1995). In normal individuals, the F-level in cerebrospinal fluid (CSF) is similar to or slightly lower than blood (Yu-Huan and Si-Shung, 1988).

The human pineal is outside the blood-brain barrier. It is one of the circumventricular organs, i.e., highly vascular, specialized neurohaemal areas of the brain which are devoid of a blood-brain barrier. The incomplete barrier is due to the absence of tight junctions occluding the inter-endothelial cell clefts in the capillaries. There is free exchange of molecules between blood and adjacent brain. Pineal capillaries are fenestrated with endothelial

cells resting on a tenuous and sometimes incomplete basal laminae (Gray's Anatomy, 1995).

There are interspecies differences with regard to the completeness of the blood-pineal barrier (Reiter, 1981). Studies have shown that the gerbil pineal is outside the blood-brain barrier (Matsushima and Reiter, 1975; Welsh and Beitz, 1981); as is the mouse pineal (Møller *et al*, 1978).

1.8 The Human Pineal Gland

The human pineal gland is situated near the anatomical centre of the brain: in the caudal epithalamus, just above the mid-brain tegmentum, occupying the depression between the superior colliculi of the mesencephalon. It is a mid-line, solid, cone-shaped, reddish-grey organ about 8 mm in length and 4 mm in greatest width. The pineal is small relative to total body weight and weighs 95 ± 49 mg, (n = 168) ranging from 18-346 mg (Hasegawa *et al*, 1987).

The base of the pineal is attached to the brain by a short stalk or peduncle, which divides anteriorly into two laminae: the superior and inferior lamina which respectively contain the habenular and posterior commissures. The pineal organ is intimately related to the third ventricle by its proximity to: (i) the pineal recess, a small ependymal lined recess of the third ventricle separating the two laminae; (ii) the suprapineal recess, a diverticulum of the ventricular roof. The major part of the pineal is covered by tissue of the tela choroidea of the third ventricle. It lies within the

subarachnoid space and is bathed by subarachnoid CSF at its outer aspect. It is surrounded by a thick fibrous capsule.

The human pineal contains cords or follicles of pinealocytes and neuroglial cells, amongst which ramifies a rich network of capillaries and postganglionic nerve fibres. The pinealocytes form the pineal parenchyma. They are polyhedral-shaped cells with long, dendrite-like processes which intermingle with the processes of neighbouring cells and often terminate in slightly expanded end-feet in association with the endothelium of the capillaries. The pinealocytes contain an abundance of cytoplasmic organelles (extensive Golgi apparatus, rough and smooth reticulum) indicative of a highly active or secretory tissue. The human foetal pinealocyte also possesses all the organelles necessary for hormone synthesis (Møller, 1974). The neuroglial cells resemble astrocytes and appear to have a supportive function for the pinealocytes.

1.9 Pineal Physiology

The mammalian pineal gland is an endocrine organ which acts an intermediary between the environment and the endocrine system. It functions as a neuroendocrine transducer. It accepts environmental sensory information (especially the photoperiod from the retinae) and converts this neural message into hormonal products, notably melatonin.

Melatonin (MT), colloquially called the hormone of darkness, is produced almost exclusively during the dark phase of the 24-hour period. Melatonin is probably a unique hormone in that its

circadian rhythm has the same phase relationship to the day-night cycle in all species; irrespective of whether the animal is diurnal, nocturnal or crepuscular. Plasma MT levels remain high and in proportion to the length of the dark phase. This rhythmicity may be important to the action of MT. The inherent rhythmicity of MT synthesis persists in the absence of a LD cycle although it runs slightly out of phase with the 24-hour cycle.

The secretion of MT is determined by an interaction between environmental lighting and the endogenous rhythmicity of the suprachiasmatic nuclei (SCN) of the hypothalamus. The onset of darkness causes information to travel from SCN (via the superior cervical ganglia) to postganglionic sympathetic nerves which terminate in the vicinity of the pinealocytes. Noradrenaline is released into the synaptic cleft where it has access to adrenergic receptors on the pinealocyte membranes. An interesting feature of pineal indole metabolism is that it is mainly controlled by the autonomic nervous system even though the pineal is part of the CNS, both anatomically and embryologically.

Pineal synthesis of MT decreases when the subject is exposed to light (even briefly) during the dark phase. Otherwise, the MT cycle is relatively resistant to other influences although it may change slightly with the oestrous cycle or be modified by nutritional (Brown *et al*, 1987) or endocrine factors (Cardinali *et al*, 1987). Acute adverse stimuli do not usually alter MT values which indicates that MT is not an index of general sympathetic activity and suggests that sympathetic control of the pineal is partitioned separately (Vaughan, 1984). The enzymatic, physiological and

rhythmic activity of the pineal cease if the neural connections between SCN and the pineal gland are destroyed or surgically interrupted following superior cervical ganglionectomy (SCGX).

There are inter-species differences in the rhythm of MT production: in the amplitude of the nocturnal MT peak, the duration of nocturnal secretion, the timing or phase relationship of the peak to the LD cycle or other physiological rhythms. The different nocturnal profiles of MT have been provisionally classified into three types. Some species, including the Mongolian gerbil, have a discrete MT rise late in the dark period and MT levels during the early hours of the dark phase are similar to those during daytime (type I). In other species, including the human and rat, MT production begins to increase at or shortly after the onset of darkness and values peak near mid-dark (type II). Thirdly, some species secrete MT soon after lights-off and values reach a plateau which is maintained virtually right through the night (type III pattern), e.g., Djungarian hamster, sheep, (Reiter, 1987).

In animals, the pattern of MT production and consideration of whether the duration of elevated MT is increasing or decreasing are important to the pineal's physiological functions. The sensitivity of the MT target tissues may also change with alterations in day length, causing different responses even in the absence of changes in MT production.

The nocturnal rise in the levels of plasma MT means that every organ in the body is aware of the environmental photoperiod. The levels of plasma MT are used as an internal reflection of the time of

day and time of year. Any changes in the pattern of MT secretion enable animals to anticipate future climatic conditions. Reproductive physiology in photoperiodically-dependent rodents is linked inextricably to the physiology of the pineal. Melatonin influences the reproductive system, either initiating or suspending reproductive function, which ensures that offspring are born in conditions most conducive to their survival. Melatonin regulates a large number of organs and systems in animals, e.g., ensuring that seasonal hibernation and pelage growth are properly timed.

In humans, there is growing evidence that MT has effects on oncogenesis, the immune system, mood and sleep disorders, jet-lag, pubertal development and the processes of both aging and age-related diseases.

1.10 Factors Suggesting that the Pineal Could Retain Fluoride

1.10.1 Pineal Calcification

The human pineal has been called the fifth mineralizing tissue since it is one of the rare tissues in the body (along with the choroid plexus) where calcification occurs physiologically. It is a well-known feature and is considered to be a 'normal' phenomenon because it is not usually associated with clinical symptoms. The calcified pineal is used as a landmark in skull X-rays because of its radio-opacity. Pineal calcification (PC) also occurs in rodents, birds and primates although there are inter-species differences. Of the common laboratory animals, the Mongolian gerbil consistently forms PC. See Section 1.16. In the

literature, pineal calcification is referred to as concretions, calcospherulites, acervuli, psammoma bodies, brain sand or corpora arenacea.

The fact that individuals vary in the extent of PC is puzzling. Sometimes there is little calcification in old age and considerable accumulation in children (Arieti, 1954). Areas of mineralization are nearly always found in adult human pineals, ranging from a fleck to almost complete replacement of the gland.

Early investigators regarded PC as a sign of degeneration and speculated that the pineal was a vestigial organ in adults. Pelham *et al* (1973) reported an inverse relationship between PC and MT in five humans. This was not confirmed by Commentz *et al*, (1986) or Bojkowski and Arendt (1990). However, the correlations between MT and PC in these studies were established on the basis of radiological evidence and not quantitatively. Skull X-rays would not demonstrate subtle areas of calcification in the pineal.

The assumption that the PC deposition lessens the pineal output of MT is not supported by histological evidence. The presence of PC did not affect the pinealocytes nor the cellularity since large numbers of pinealocytes were present in pineals from subjects from puberty to old age with maximum cellularity for both sexes in the 30-44-year-age group (Tapp and Huxley, 1971; 1972). The pinealocytes from young children appeared histologically similar to aged subjects (Rodin and Overall, 1967). The presence of PC

did not affect enzyme activities in human pineals from infancy to old age (Wurtman *et al*, 1964).

The human pineal gland does not atrophy with increasing age nor should its calcification be regarded as a sign of degeneration. On the contrary, there is evidence that the presence of calcification within the pineal reflects its past metabolic activity.

Interruption of the sympathetic innervation of the pineal by SCGX prevented the normal accumulation of PC and vacuoles in the superficial pineal gland of young gerbils (Reiter *et al*, 1976); and significantly reduced the number of PC in the pineals of adult male gerbils (in which PC had already formed) (Champney *et al*, 1985). Concretions may be labile structures which are maintained directly or indirectly by an intact sympathetic innervation. Blocking the β-adrenergic stimulation of the gerbil pineal at four weeks (by daily afternoon sc injections of propranolol) caused a significant reduction in the number of vacuoles and PC eight weeks later (Vaughan *et al*, 1986).

Extremes in lighting conditions also influence PC formation. Gerbils under constant light had reduced numbers of PC (Welsh, 1977); under short photoperiods had increased numbers of PC (Lewinski *et al*, 1983). Darkness stimulates the biosynthetic activity of the gland and ultimately leads to the formation of PC whereas constant light inhibits their formation. The number of PC in gerbils was decreased following the chronic administration of MT, (either as sc MT pellets, which released the indole

continuously, or as daily afternoon sc injections) (Vaughan *et al*, 1983).

The concretions are found throughout the pineal gland: primarily in the glial and stromal compartments, and often lying in the immediate vicinity of blood capillaries (Galliani *et al*, 1990). When they lie immediately adjacent to the pinealocytes, there is no evidence of a reaction to the PC nor compression of the pinealocytes (Tapp and Huxley, 1972). The concretions appear to originate intracellularly in the cytoplasm of the pinealocytes (in vacuoles) by deposits of 'calcareous material'. They continue to grow in size either within the vacuole or extracellularly and after reaching some undetermined stage of development, they are extruded into the extracellular matrix: in humans (Cooper, 1932; Allen *et al*, 1981); in gerbils (Welsh and Reiter, 1978; Welsh, 1985).

The concretions appear to be multilobulated as though they consisted of small, round, milky-white marbles which had been glued together to form an acinar, mulberry-shaped configuration. The concretions vary in size from small microscopic granules to large bodies showing concentric laminations, i.e., from 300 to 3000 μm, (Krstic, 1976; Allen *et al*, 1981). Fractured un-decalcified concretions expose a glass-like surface and the mulberry-like surface is not repeated within the concretion (Earle, 1965; Krstic, 1976; Allen *et al*, 1981). Decalcified concretions show an irregularly arranged material and sometimes concentric lamellae (Kristic, 1976).

Calcareous concretions are formed on an organic matrix which is synthesized by the pinealocytes. Organic substances were identified as carbohydrates, probably acid mucopolysaccharides complexed to a protein (Japha *et al*, 1976; Humbert and Pévet, 1991). A progressive stratification leads to an increase in size. They are concentrically microlaminate structures in which protein-rich zones alternate with apatite-rich zones. They are composed of aggregates of needle-shaped, randomly orientated crystals, 300-500 Å long and 25-35 Å thick (Krstic, 1976); 200-220 Å long and 35-45 Å wide (Mabie and Wallace, 1974).

The main component of the mineral deposited in PC is hydroxyapatite (Angervall *et al*, 1958; Earle, 1965; Mabie and Wallace, 1974; Krstic, 1976; Galliani *et al*, 1990; Bocchi and Valdre, 1993)). Carbon dioxide is evolved by acid treatment which suggests that the apatite mineral contains carbonate (Earle, 1965; Mabie and Wallace, 1974). Small amounts of ß-tricalcium phosphate are also present (Galliani et al, 1990). The chemical composition, morphology of crystals and unit cell dimensions of PC were found to be comparable with those of HA deposited in bone tissue and tooth enamel (Mabie and Wallace, 1974). The calcium to phosphorus ratio was estimated as 1.586 (Earle, 1965); 1.595-1.615 (Mabie and Wallace, 1974); 1.53 (Galliani *et al*, 1990); 1.65 (Bocchi and Valdre, 1993). The low calcium to phosphorus ratio (as compared to the theoretical 1.667) is typical of biological hydroxyapatites elsewhere in the body and may be caused by trace elements replacing calcium in the crystal lattice.

Pineal concretions contain high levels of trace elements including: magnesium (Krstic, 1976; Michotte *et al*, 1977; Allen et al, 1981) sulphur (Humbert and Pévet, 1991) and strontium (Krstic, 1976; Allen *et al*, 1981); manganese (Michotte *et al*, 1977); zinc and copper (Michotte *et al*, 1977; Humbert and Pévet, 1991). The levels of these trace elements usually lie within the limits found in other biological apatites (Michotte *et al*, 1977).

Uncalcified human pineals also contain high levels of trace elements (Michotte *et al*, 1977); so do uncalcified rat pineals (Humbert and Pévet, 1991). This may be due to the presence of very small calcium particles within the pineal which 'trap' the trace elements by continuous ion transfer and ion exchange even though the calcification would not be detectable using conventional methods. Such an explanation would be consistent with the accepted theory of the ontogeny of concretions, i.e., the formation of very small particles which coalesce to form larger particles. In which case, uncalcified pineals are simply at an earlier stage of calcification and the characteristic concentric laminations of PC have not yet formed. The calcification would not be visible on skull X-rays.

1.10.2 Prevalence of Pineal Calcification in Humans

The incidence of PC in different age groups has been estimated using conventional skull radiography and histological examination of autopsy specimens. There is limited paediatric material because, under normal circumstances, the young do not

present for radiological or post-mortem examinations. The incidence of PC is very high in humans. Some of the early radiographical studies gave the impression that PC does not occur in early life and only appears after puberty. Large amounts of PC have been demonstrated in children and some aged glands have virtually none (Cooper, 1932; Arieti, 1954; Galliani *et al*, 1990). Significant amounts of calcification were found in the small number of pineal glands from children aged 0-5-years using the difference in the weight of the pineal before and after decalcification (Tapp and Huxley, 1971).

Histological examination is the most sensitive method of detecting the small, punctate calcification in the child's pineal. The ground-substance matrix in which PC originates was demonstrated in pinealocytes from infants soon after birth (Wurtman, 1968); PC were present in eight out of 28 pineal glands from children under the age of one year (Heidel, 1965); PC formed in children aged three years (Kerényi and Sarkar, 1968); Doskocil (1984) found PC in an infant aged one year. Four out of five human pineals from young subjects in the second decade contained concretions and 'one gland contained such quantities that sectioning was extremely difficult' (Cooper, 1932).

It has been estimated that concretions must have aggregated to a critical size (4×10^{-5}m in diameter) before they are visible on X-ray (Doskocil, 1984). Therefore, skull radiography is the least sensitive method of detecting PC even when the radiographs are of excellent quality. Microscopic calcific crystals are present in

all pineal glands and calcification of the pineal is more frequent than has been previously estimated (Michotte *et al*, 1977).

The widespread knowledge about the incidence of PC in the older age groups was largely obtained using conventional skull radiography, i.e., a rising incidence in PC with increasing age. However, a significant incidence of PC has also been noted in the first decade of life using X-rays: 3.7% of children between 0 to 6 years of age, 5.5% in the 7 to 8-year-old group and a substantial increase after 9 years (Winkler and Helmke, 1987). On the other hand, several studies failed to detect PC in this age group (Adeloye and Felson, 1974; Bhatti and Khan, 1977; Daramola and Olowu, 1972).

Cranial computed tomography (CCT) is a more sensitive method of detecting PC than skull X-rays. Using CCT, a 2% and 32% incidence of PC in the first and second decades respectively (Macpherson and Matheson, 1979); a 0.2% incidence of PC in the 0-5-year age group, 5.8% in the 6-10-year age group and 14% in the 11-15-year age group (Ando *et al*, 1987); 8-11% of children between 8-14 years; 30% at 15 years and 40% at 17-18 years (Zimmerman and Bilaniuk, 1982).

There is the tendency for the calcification to become progressively more aggregated and consequently more radiographically detectable at puberty. Pineal calcification may be related to the onset of puberty (Winkler and Helmke, 1987; Macpherson and Matheson, 1979; Zimmerman and Bilaniuk, 1982).

1.10.3 Blood Supply

The degree of vascularity within a mineralized tissue governs F-uptake (WHO, 1970). One of the most outstanding features of the pineal, noted even by the early anatomists, is the obvious, conspicuous capillary network within the pineal gland. The blood supply is profuse even though the pineal is small relative to the total body mass. The foetal pineal also receives a copious blood supply (Møller, 1974). The minimum rate of rat pineal blood flow per gram exceeds that of most endocrine organs, equals that of the neurohypophysis and is surpassed only by that of the kidney (Goldman and Wurtman, 1964). The blood supply to the pineal is greater at night than during the day which is related to the night-time rise in indole metabolism (Reiter, 1981).

In conclusion, although the exact incidence of PC in children is unknown, it is likely that the child's pineal has the capacity to sequester F from the circulation.

1.11 Melatonin Synthesis

Pinealocytes actively take up the essential amino acid, tryptophan, from the systemic circulation and form a variety of indolic intermediates and products. Serotonin (5HT), the precursor of melatonin, is synthesized from tryptophan in a two-step reaction. Firstly, tryptophan is hydroxylated in the 5 position to form 5-hydroxytryptophan by the mitochondrial enzyme, tryptophan-5-hydroxylase. Secondly, 5-hydroxytryptophan is decarboxylated by

the cytoplasmic enzyme, 5-hydroxytryptophan decarboxylase, to form serotonin (5-hydroxytryptamine, 5HT).

The concentration of serotonin in the pineal (and the activity of 5-hydroxytryptophan decarboxylase) are high compared to other regions of the brain. They are greater during the day than at night, i.e., 180° out of phase with the levels of other pineal amines and their associated enzyme activities. About a half of the serotonin synthesized by the pinealocytes is released and taken up by adjacent noradrenergic nerve endings. Serotonin is metabolized by three major routes within the mammalian pineal.

The best known pathway is its conversion to melatonin by the sequential activities of two enzymes. Initially, serotonin is converted to *N*-acetylserotonin (NAS) by the enzyme serotonin *N*-acetyltransferase (NAT) using acetyl CoA as the acetyl donor. NAT is the rate-limiting enzyme in the synthesis of MT from 5HT. Its activity exhibits a 24-h rhythm with higher values during the night. NAT has received the most attention in the literature because the stimulus for the nocturnal increase in MT synthesis arises from the increase in NAT activity (which is activated by the release of noradrenalin on ß-adrenergic receptors in the pinealocyte membrane). NAS is converted to melatonin (*N*-acetyl-5-methoxytryptamine, MT) by the cytosolic enzyme, hydroxyindole-*O*-methyltransferase (HIOMT). See Figure 1.3.

Secondly, pineal serotonin can be metabolized by the mitochondrial enzyme, monoamine oxidase, to 5-hydroxyindole acetaldehyde, an unstable product which is either oxidized to 5-

hydroxyindoleacetic acid or reduced to 5-hydroxytryptophol. Both compounds are *O*-methylated by HIOMT to form 5-methoxyindole acetic acid and 5-methoxytryptophol, respectively.

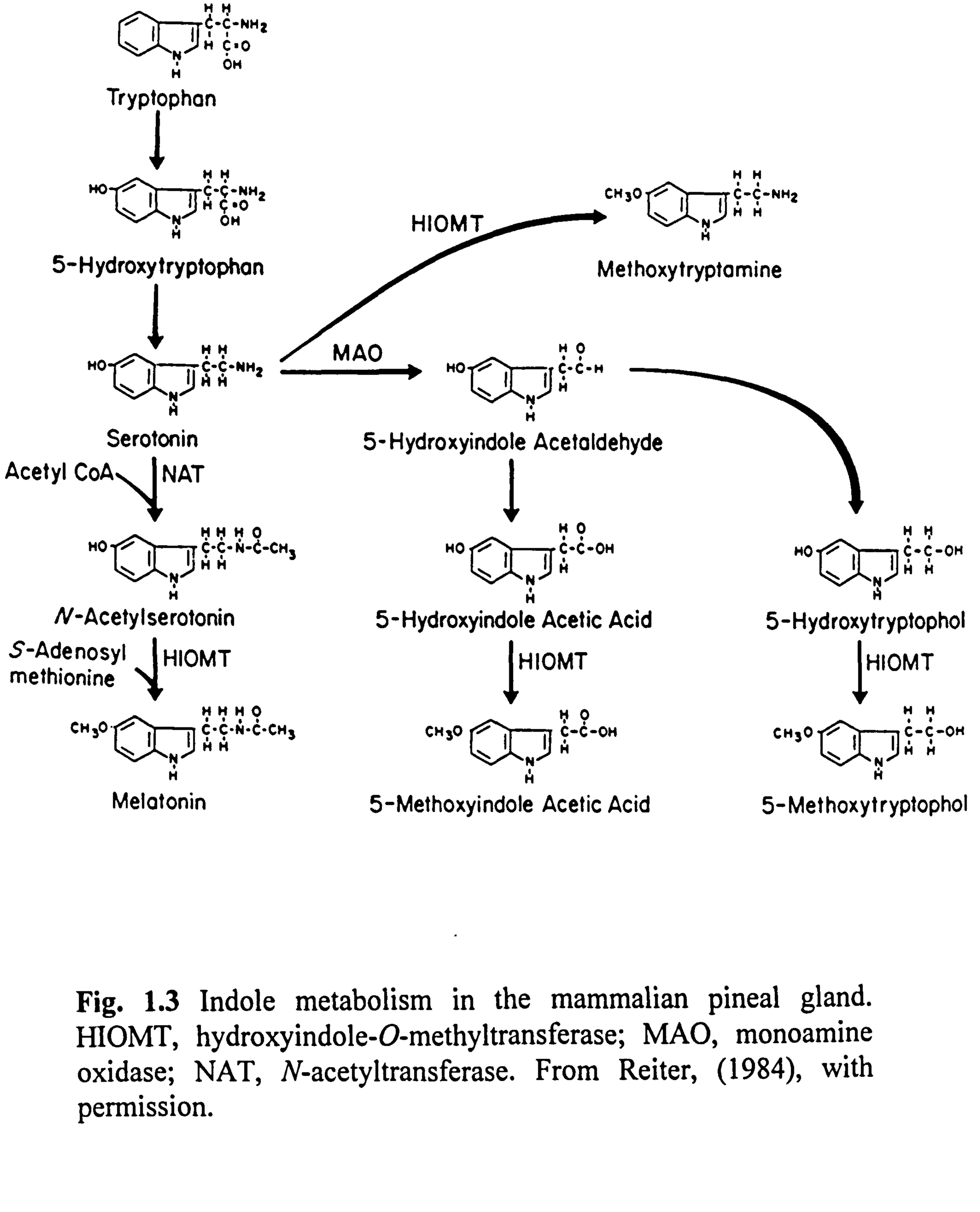

Fig. 1.3 Indole metabolism in the mammalian pineal gland. HIOMT, hydroxyindole-*O*-methyltransferase; MAO, monoamine oxidase; NAT, *N*-acetyltransferase. From Reiter, (1984), with permission.

Thirdly, the enzyme, HIOMT, directly methylates pineal serotonin to 5-methoxytryptamine, a potential pineal hormone. (See reviews by Arendt, 1995 and Reiter, 1991).

This pattern of tryptophan metabolism is not the prerogative of the pineal gland. It also occurs in the retina, Harderian and lacrimal glands. These extra-pineal sites of MT production are usually associated with the visual system. Melatonin synthesized by these tissues presumably has local effects since it is not discharged into the general circulation. Pinealectomized animals have non-detectable plasma levels of MT (Arendt, 1985).

This simplified account of indole metabolism does illustrate the complexity of interactions operating within the pineal gland. At least four active methoxyindoles exist in the human pinealocyte (Beck *et al*, 1982). Some are potential pineal hormones in their own right, e.g., 5-methoxytryptamine has similar endocrine effects as MT (Reiter, 1989). The pineal also produces peptidergic compounds, e.g., arginine vasotocin.

Fluoride inhibits numerous enzyme reactions, especially those requiring divalent cations. There are a number of calcium-dependent steps in the biosynthesis and release of MT. On a molecular level, calcium has inhibitory or activating properties on various enzymes which balance catalytic activity. (See review by Morton and Reiter, 1991). Fluoride could affect the biosynthesis of MT by binding with calcium. With this in mind, it would seem naive to assume that F does not influence one or more of the enzymes in the pathway from tryptophan to MT.

1.12 Melatonin

Melatonin is a small (MW 232) highly lipophilic hormone which easily penetrates cell membranes to gain access to every fluid and presumably every cell in the organism. It crosses the placenta to the foetus, is transferred via breast milk to the infant, and crosses the blood-brain barrier into the brain. About 70% of circulating MT is bound to albumin. The concentrations of MT in body fluids, e.g., blood, cerebrospinal fluid, urine, saliva, lymph, seminal fluid, breast milk and amniotic fluid, exhibit a diurnal rhythm which coincides with pineal MT output, i.e., highest levels during the night-time. The pineal does not contain large reserves of MT and the hormone is probably released directly into the blood stream and only secondarily into other body fluids (Reiter, 1986). The specific release mechanisms for MT from the pinealocytes are not poorly understood.

1.13 Metabolism of Melatonin

Circulating melatonin is rapidly metabolized in the liver and the metabolites are subsequently excreted in urine. Several studies have shown that the predominant metabolic pathway of MT is the hydroxylation on the six carbon position by liver enzymes to form 6-hydroxymelatonin, which in turn is conjugated to form 6-sulphatoxymelatonin (aMT6s) and the glucuronide conjugate respectively. In humans, 60-80% of administered radiolabelled MT was excreted as aMT6s and 13-27% as glucuronide conjugate (Jones *et al*, 1969; Young *et al*, 1985). In mice, 70% of administered radiolabelled MT was excreted as urinary aMT6s, and

6% as glucuronide conjugate (Kopin *et al*, 1961). In rats, 55% was excreted as aMT6s and 30% as glucuronide conjugate (Kveder and McIsaac, 1961). The relative amounts of sulphate and glucuronide conjugates depend on the species (Arendt, 1995). In addition, exogenous MT can be demethylated to produce *N*-acetylserotonin, the biosynthetic precursor of MT, and excreted as sulphate or glucuronide conjugates in rats, (Leone and Silman, 1984); and in humans (Young *et al*, 1985). Urine contains less than 1% of free, unmetabolized MT (Kopin *et al*, 1961).

1.14 Melatonin Production During Pubertal Development

The amount of MT produced by the pineal varies throughout the human life span. The nocturnal levels of plasma MT are highest during early childhood and decline during puberty (see below); at around middle-age, the levels of plasma MT decline further and progressively until senescence, particularly with regard to the peak values and duration (Iguchi *et al*, 1982). The fact that the nocturnal MT levels are highest in the young and markedly decline with age suggests that the pineal and its hormone, MT, have specific functions during childhood. If MT is involved in the process of sexual maturation, one would expect the MT levels in body fluids of prepubertal children to differ from those in adults.

At the turn of the century, the coincidence of pineal tumours and precocious puberty in humans initiated many animal studies on the pineal/puberty hypothesis. In seasonally breeding species, the pineal gland and MT have a modulating effect on sexual maturation and reproductive function. Humans are not seasonal

breeders and the interaction between the pineal and sexual function is less clear. Nevertheless, the available animal and clinical data suggest that changes in pineal MT secretion may be involved in the onset of human puberty. One of the main trends in pineal research is to investigate the role played by the pineal gland during sexual maturation in humans. It remains a controversial topic.

1.14.1 Human Studies

Several studies investigating the changes in MT secretion during normal human puberty used the MT levels in plasma collected during the daytime and found no significant differences between the levels of MT in daytime plasma in children at different stages of pubertal development (Lenko *et al*, 1982; Ehrenkranz *et al*, 1982; Gupta *et al*, 1983; Waldhauser *et al*, 1984). One study (Silman *et al*, 1979) reported that prepubertal boys had significantly higher daytime plasma levels of MT than pubertal boys. However, MT levels are very low, even non-detectable, in daytime plasma and present technical difficulties in accurately measuring them. Besides, an evaluation of daytime MT levels during puberty may have little physiological significance.

Since most MT is secreted during the dark phase, studies which used MT levels in plasma collected during the night are more informative. Unfortunately, they are often contradictory. Many studies support a relationship between MT and pubertal development. The levels of MT in night-time plasma were reported to be highest in the pre-school child and thereafter

decline progressively and significantly throughout the stages of sexual development (Tamarkin *et al,* 1982; Gupta *et al,* 1982; 1983; Lissoni *et al*, 1983; Waldhauser *et al*, 1883; 1984; 1988; Attanasio *et al*, 1985). There were studies, however, in which no significant differences were found between the levels of MT in night-time plasma from children at various stages of pubertal development (Ehrenkranz *et al*, 1982; Tamarkin *et al*, 1982; Sizonenko *et al*, 1985).

A more integrated picture of the total amount of MT secreted by the pineal in 24-hours can be obtained by measuring the amount of the MT metabolites excreted in the urine in 24-hours. No correlation was found between the daily excretion rates of conjugated 6-hydroxymelatonin and age in children aged 3-16 years (Tetsuo *et al*, 1982) The rate of urinary melatonin metabolites remained constant during childhood and puberty (Sizonenko *et al*, 1985; Young *et al*, 1988; Bojkowski and Arendt, 1990; Rager *et al*, 1989). These studies suggest that the pineal secretes a constant amount of MT during childhood.

However, rates of excretion of urinary MT metabolites need to be expressed as a function of body weight in order to reflect the circulating concentration of MT. This conversion is particularly necessary in paediatric studies where body weights can vary considerably. As mentioned earlier, it has been proposed that the pineal produces a constant amount of MT during childhood and the exponential decrease in peripheral MT concentrations is due to increasing body weight with age. Increasing body size

obviously dilutes the circulating MT and leads to a reduction in the concentration of plasma MT (Waldhauser *et al*, 1988; Young *et al*, 1988).

When the excretion rates of urinary aMT6s are expressed as ng aMT6s/h/kg BW, studies have shown that the highest rates of aMT6s are present in the youngest age group and the excretion gradually decreases throughout puberty and adolescence whereas nocturnal aMT6s output remains fairly constant (Young *et al*, 1988; Bojkowski and Arendt, 1990; Rager *et al*, 1989). This assumes, of course, that the proportion of MT which is metabolized to the metabolite aMT6s also remains constant with increasing body weight.

1.14.2 Animal Studies

Pineal concentrations of MT, plasma MT and NAT were studied in 3-, 8-, and 68-week-old male rats. At mid-dark, the pineal MT contents in adult and prepubertal rats were similar and both were higher than in the senile rat. When expressed in terms of body weight, the prepubertal rat had the highest MT levels and the senile rat the lowest levels. Similar results were obtained with the serum MT. When NAT activity was expressed in terms of body weight, the prepubertal rat had the highest values of NAT activity and the lowest values were found in the senile rat pineal. These results show a clear-cut, age-related reduction in pineal NAT and pineal MT with highest values in the prepubertal rat and lowest in the senile rat. The levels of pineal MT during the day were about the same for

all ages (Pang *et al*, 1984). Pineal and plasma MT increased with sexual development in the rat (Pang *et al*, 1990; Tang and Pang, 1988).

Melatonin production in animals is gradually lost throughout life: in very old individuals the circadian MT rhythm is barely discernible. This has been demonstrated in the rat (Pang *et al*, 1984; 1990; Reiter *et al*, 1981; Yie *et al*, 1992); in the Syrian hamster and gerbil (Reiter *et al*, 1980).

1.14.3 Sex Differences

No sex difference was found in the concentration of serum MT in humans (Waldhauser *et al*, 1984; 1988); in total aMT6s excretion by humans aged 2 to 80 years (Bojkowski and Arendt, 1990); in pineal contents of MT and serotonin in rats (Wakabayashi *et al*, 1986); in Syrian hamsters (Lasley *et al*, 1984). On the other hand, a sex difference in aMT6s excretion was reported in rats (Yie *et al*, 1992). Sex differences in pineal morphology were noted in humans (Blumfield and Tapp, 1974).

1.15 Measurement of Pineal Function

Pineal function in large species, e.g., humans and domestic animals, has been studied by taking repeated blood samples for the subsequent determination of plasma MT levels. However, small laboratory animals such as rodents have rather small blood volumes and blood vessels which are not easily cannulated. Therefore,

knowledge on pineal MT synthesis in rodents has been acquired by sacrificing the animals either to obtain sufficient blood for assaying plasma MT or to remove the pineal glands throughout the entire 24-hour cycle. The daily profile of pineal MT content or pineal NAT activity is then measured in vitro. The activity of NAT can be used as a biochemical parameter of pineal metabolic function since pineal NAT has a rate-limiting role in controlling MT synthesis. However, experiments requiring pineal gland assays preclude longitudinal studies of individual animals. They also require the sacrifice of hundreds of animals because one animal only provides one data point at best.

For obvious reasons, a definition of the pineal MT rhythm in humans can only be inferred from the 24-h rhythm of blood MT. Since a high degree of correlation between these two cycles has been demonstrated in other mammals, there is every reason to believe that this also exists in humans. Several human studies have demonstrated that the pronounced pattern of secretion of pineal MT as shown by plasma MT levels throughout the 24-hour cycle is in turn reflected in the levels of aMT6s excreted in urine (Markey *et al*, 1985; Arendt *et al*, 1985; Nowak *et al*, 1987). Therefore, the measurement of the excretion rates of urinary aMT6s is a reliable index of plasma MT concentrations in humans and a convenient, non-invasive method of obtaining an indirect assessment of pineal MT synthesis and its circadian rhythm.

Experimental paradigms with well-established effects on pineal MT production have been applied to animal studies to discover whether the rates of metabolite excretion mirror the predicted

changes in pineal MT production. These experimental paradigms include: a) alterations in the lighting schedule in rats (Brown *et al*, 1991; Kennaway, 1993); in Rhesus monkeys measuring conjugated 6-hydroxymelatonin (Tetsuo *et al*, 1982); b) ganglionectomy in the mink (Maurel *et al*, 1992); c) pinealectomy in the rat (Markey and Buell, 1982; Brown *et al*, 1991); in the Djungarian hamster (Stieglitz *et al*, 1995); and in Rhesus monkeys measuring conjugated 6-hydroxymelatonin (Tetsuo *et al*, 1982). Peak nocturnal pineal contents were significantly correlated with 24-h urinary aMT6s excretion in the Djungarian hamster (Stieglitz *et al*, 1995). These studies concluded that rates of urinary aMT6s excretion can be used as an accurate index of pineal MT synthesis.

Besides being a non-invasive technique, the advantages of using levels of urinary aMT6s to assess pineal function are: (a) the number of subjects required is low compared with alternative approaches such as the measurement of pineal MT or pineal enzyme contents (animal studies); (b) the 24-hour pattern of pineal activity can be assessed in individuals; (c) pineal function can be monitored over a period of time (weeks or months) in the same individual; (d) additional physiological and morphological parameters can be monitored simultaneously; (e) facilitates studies on the influence of different factors, such as drugs, menstrual cycles, age, etc, on MT secretion; (f) the daily urinary excretion of aMT6s reflects the total amount of MT secreted by the pineal whereas measurements of plasma and pineal MT levels provide a momentary pineal MT concentration; (g) methodology is simple since the concentration of urinary levels of the MT metabolites are 1000-fold higher than urinary MT.

1.16 Animal Model: the Mongolian Gerbil, *Meriones unguiculatus*

The Mongolian gerbil (genus Meriones, subfamily Gerbillinae of the family Cricetidae, order Rodentia) is a small, desert rodent whose natural habitat is the semi-arid areas of North-East China and Eastern Mongolia.

Fig. 1.4 Family of Mongolian gerbils, *Meriones unguiculatus*

The gerbil has been used as the animal model for physiological research on water conservation because they need very little water compared to other common laboratory animals. Their voluntary daily water consumption was 0.04 ml/g BW (Winkelmann and Getz, 1962; Arrington and Ammerman, 1969). Gerbils can survive without water for more than 45 days provided they are not stressed (Boice and Witter, 1970). Consequently, they excrete very small

quantities of urine. Gerbils do not hibernate or aestivate and are naturally active during night and day (Robinson, 1959).

The gerbil is often used as the animal model in pineal research. It was selected for use in this study because, unlike other non-human species, the gerbil pineal consistently contains PC. At three weeks of age, (the age of weaning), PC was identified within the pinealocytes in the superficial part of the gerbil pineal in a third of the subjects; by eleven weeks of age, (time of sexual maturity), all gerbil pineals contain PC (Japha *et al*, 1974, 1976).

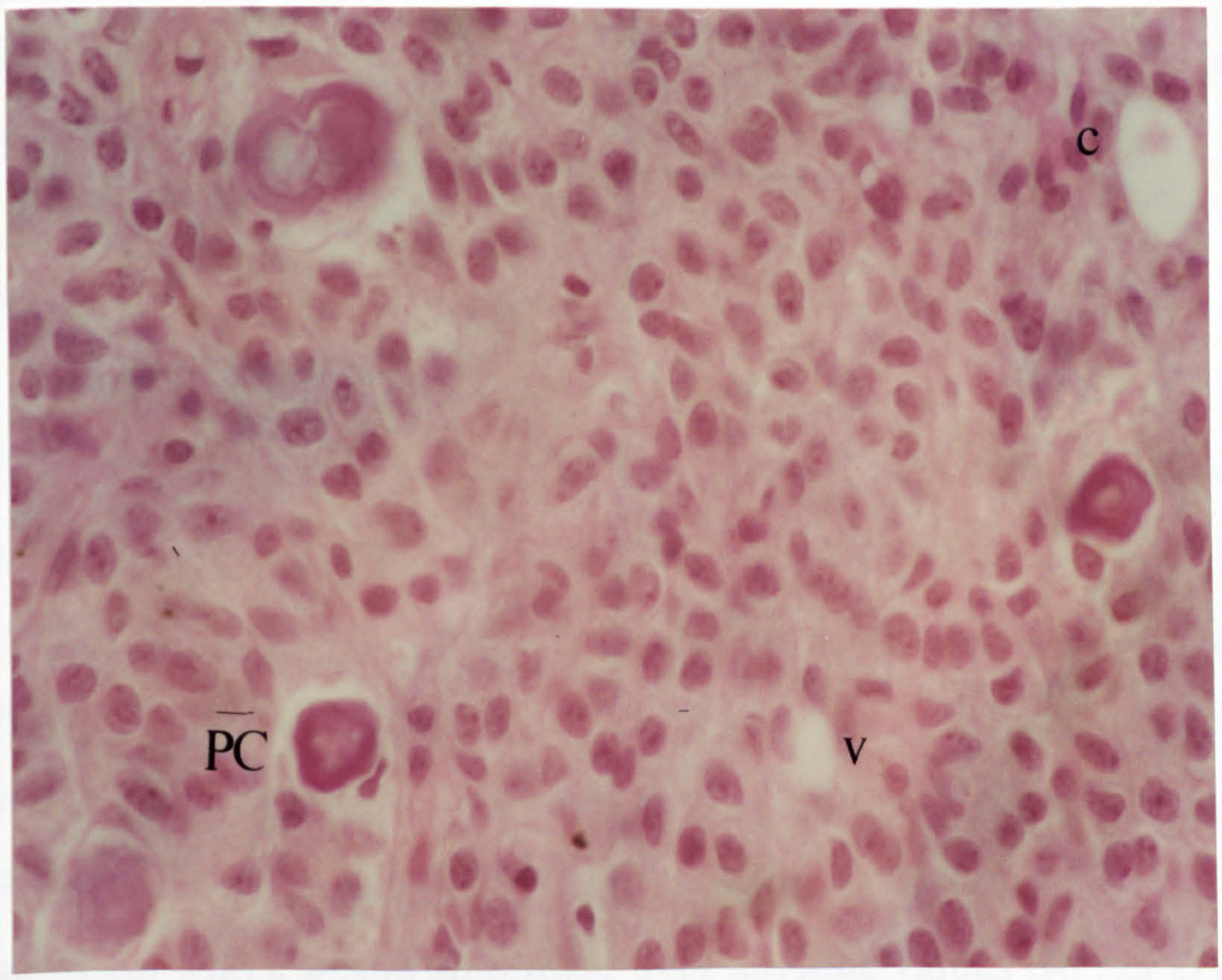

Fig. 1.5 The superficial pineal gland from a 12-week-old gerbil; fixed with Bouin's fluid, stained with haematoxylin and eosin, magnified by 200. **N.B**. The vacuole within the pinealocyte (v); the eosinophilic material within the vacuole (c); the calcified extracellular concretion showing a concentric structure (PC).

The mineral composition of the gerbil and human PC is qualitatively and quantitatively identical (Krstic and Golaz, 1977). The ontogeny, morphology and development of human and gerbil PC are similar. Concretion formation in the gerbil pineal begins with the appearance of vacuoles in the pinealocytes. The vacuoles and concretions increase in number and size throughout the life of the gerbil (Welsh and Beitz, 1981). The formation of PC by the gerbil is a normal physiological process (Vaughan, 1986) as it is in humans. Although the gerbil is an excellent model for studying the formation of PC under controlled conditions, it is less satisfactory when the study requires the collection of urine because the gerbil has low water requirements and subsequently a low rate of urine excretion.

1.17 Pineal Function in Gerbils

The zenith of pineal NAT activity in the gerbil (neither age or sex reported) occurred eight hours after lights off (0400) under LD: 14 10 with a 3-fold amplitude over daytime values (Rudeen *et al*, 1975). In male gerbils aged 8 weeks exposed to LD: 14 10, the nocturnal peak values of pineal MT contents were 225 pg MT/gland and occurred at 0400, eight hours after the onset of darkness; with a 2-fold amplitude over daytime values (Reiter *et al*, 1980). In male gerbils aged 8 weeks under LD: 14 10, the activity of pineal NAT and pineal MT content (123 ± 16 pg/gland, mean ± SEM) were highest at 0200, six hours after the onset of darkness; daytime pineal MT contents were 22 to 53 pg/pineal (King *et al*, 1981).

Electrophysiology is an important parameter of secretory activity in the mammalian pineal. The mean firing frequency in pinealocytes from male gerbils aged 3 to 5 months (under LD: 12 12, lights off at 1900) showed a diurnal rhythm with peak values occurring between 0100 to 0400, six to nine hours after the onset of darkness, with a 5-fold amplitude over daytime values (Stehle and Reuss, 1988).

1.18 Age of Sexual Maturity in Gerbils

1.18.1 Males

Sexual maturity in male gerbils was reached at about 10 to 12 weeks of age as judged by the occurrence of active spermatogenesis and mating behaviour (Marston and Chang, 1965). Testes descend at 30-45 days of age (Nakai *et al*, 1960; Schwentker, 1963; Marston and Chang, 1965). Serum testosterone concentrations significantly changed from birth to adulthood. They were maximal (4.2 ± 0.4 ng/ml) at 10 weeks of age (Probst, 1985). Norris and Adams (1972) estimated that male gerbils were 130 to 140-days-old when they sired their first litters.

1.18.2 Females

Age at vaginal opening is widely used as an index of sexual development in laboratory studies of female rodents. In the Mongolian gerbil, vaginal opening has been reported to occur at 49 days with a range of 40-76 days (Nakai *et al*, 1960); 45 days

(Schwentker, 1963); 40-60 days (Marston and Chang, 1965); 41 days with a range of 33-53 days (Norris and Adams, 1979). On the other hand, Clark and Galef (1985) reported a clear bimodality in the age at which gerbils achieve vaginal introitus: precocious and late-developing females exhibited perforation at mean ages of 16 and 35 days respectively. Female gerbils are sexually mature at 70-84 days (Schwentker, 1963); 63-84 days (Nakai *et al*, 1960; Marston and Chang, 1965); Norris and Adams, 1972b). Female gerbils reach functional sexual maturity earlier than males. The female gerbil is polyoestrous with a short oestrous cycle of about 4 to 6 days. The gestation period is 24 to 26 days.

1.19 Physiological Signs of the Onset of Puberty in Gerbils

1.19.1 Ventral Gland

The gerbil has a sebaceous, scent-marking gland in the mid-line of the abdomen, which it uses to personalize its environment. It is a discrete, wax-like, orange, fusiform pad which produces a yellowish-brown sebum of musky odour. In a well-developed male, the surface may be 3 cm long and 0.7 mm wide and somewhat less in the adult female (Thiessen *et al*, 1968). Both males and females rub the ventral gland over low-lying objects, leaving the sebum as a territorial marker. This behaviour is seen more frequently in males than females (Thiessen, 1973).

Although very little is known about the functional significance of this behaviour, it depends upon the presence of androgens in

blood (Yahr and Thiessen, 1972) and brain (Thiessen *et al*, 1973). Rat preputial and sebaceous glands have traditionally been used to study the hormonal influences on glandular development: the weight increases of the glands are a function of the amount of hormone injected and presumably of their endogenous production as well.

Fig. 1.6 The ventral gland of the Mongolian gerbil

The ventral gland is a biological index of hormonal activity. Its size is dependent on gonadal hormones which suggests that ventral gland may be an indication of gonadal development (Thiessen, 1973). The primordium of the juvenile gland appears as a barely perceptible streak in the mid-line of the abdomen. The gland rapidly develops between 4-8 weeks in male gerbils,

long before spermatogenesis, at about the time of testicular descendence (Schwentker, 1963). It develops later in females: between 8-12 weeks (Cheal and Foley, 1985). Its appearance gives a good indication of functional ovaries and uteri and is therefore correlated to sexual maturation (Swanson and Lockley, 1978). The accelerated growth of ventral glands in male and female gerbils following their brief exposure to an outdoor, desert environment was indicative of earlier sexual maturity (Cheal *et al*, 1986).

1.19.2 Body Weights

Embryological development of the gerbil foetus is comparable to that of the mouse and rat. The young are born at the same stage of development as the rat but thereafter their rate of growth is considerably slower. Duration of light exposure during ontogeny altered the rate of growth of both male and female gerbil pups (Clark and Galef, 1981).

There is a large and often contradictory literature concerning the relationship between body weight and the onset of sexual maturity in rodents. The rate of attainment of a critical weight is considered to be one index of pubertal development.

1.19.3 Testes

The response of the reproductive organs to MT treatment has been studied in gerbils. Testicular weight in gerbils was not reduced at 13 weeks of age following 6 weeks of chronic

exposure to MT-beeswax pellets (Vaughan *et al*, 1976). Testes weights and plasma-testosterone levels in five-month-old gerbils were significantly reduced following MT injections every afternoon for 12 weeks (Vaughan *et al*, 1983; Reiter 1981).

At 16 weeks, the mean paired testes weight in gerbils was 1803 ± 58 mg/100 g BW (Probst 1985).

Chapter 2 - Aims and Objectives

1. The purpose of the first experiment was to discover whether F accumulates in the human pineal gland. The objectives were to determine:

a) The [F] of human pineal gland and corresponding muscle and bone so that the pineal [F] could be compared to that of muscle and bone.

b) The [Ca] of human pineal gland so that pineal [Ca] could be correlated with pineal [F], and an estimation made of the amount of hydroxyapatite (HA) in the pineal.

2. The purpose of the second experiment was to discover whether F affects pineal physiology: specifically, its ability to synthesize melatonin (MT). The aim was to set up a controlled longitudinal study of the effects of F on the pineal output of MT during the transition from prepubescence through puberty into young adulthood using the Mongolian gerbil (*Meriones unguiculatus)* as the experimental animal model. The levels of urinary 6-sulphatoxymelatonin, aMT6s, were used as an index of pineal MT synthesis.

The objectives were:

a) To collect urine from two groups of gerbils, high-F (HF) and low-F (LF), at 7, 9, 11½ and 16 weeks of age at 3-h intervals over 48-h for the subsequent measurement of the levels of urinary

aMT6s. The levels of urinary aMT6s were determined using radioimmunoassay (RIA).

b) To validate the RIA for urinary aMT6s currently in use in the laboratory for use with gerbil urine.

c) To demonstrate that the amount of MT synthesized by the gerbil pineal reflects the excretion rate of aMT6s in gerbil urine in 16-week-old gerbils. The aims were to determine the gerbil pineal MT contents at 6-h intervals over 24-h and the excretion rates of urinary aMT6s at 3-h intervals over 24-h using RIAs. The pineal MT/urinary aMT6s relationship was assessed: (i) qualitatively, by comparing the circadian profiles of pineal MT content and urinary aMT6s excretion by 16-week-old gerbils; (ii) quantitatively, by correlating peak nocturnal pineal MT content with total urinary aMT6s pg/g BW/24-h.

d) To compare the rate and pattern of urinary aMT6s excreted by the HF and LF groups during sexual maturation.

e) To compare the circadian profiles of urinary aMT6s by the HF and LF groups at 11½ weeks (sexual maturity) and at 16 weeks (adulthood) in order to discover whether F affects the rhythmicity of urinary aMT6s excretion, e.g., the amplitude, the time of appearance and decline of urinary aMT6s excretion, and the total amount of urinary aMT6s excreted during the daytime and night-time.

3. The purpose of the third experiment was to discover whether F affects the timing of the onset of sexual maturation in gerbils. The objective was to compare several physiological markers for the onset of puberty in the two groups, i.e., the areas of the ventral glands, age of vaginal opening, body weights and weights of testes.

4. The purpose of the fourth experiment was to demonstrate that F was the only variable between the two groups. The aim was to compare the [F] of gerbil bone ash from the HF and LF groups at various ages.

The project will provide basic information on the rate and circadian profiles of urinary aMT6s excretion during the development of the gerbil, a common species in pineal research. Such basic knowledge is a prerequisite for further studies using urinary aMT6s measurements as an alternative to pineal or plasma MT determinations in the gerbil. It was hoped that the results would contribute new knowledge on pineal MT output during puberty in gerbils and contribute towards knowledge about the pineal's function during sexual development.

The results will add new knowledge about the fate and distribution of F in the human body. Although it is difficult to evaluate the relevance of gerbil data to the human situation, the results may suggest a relationship between F and the timing of the onset of human puberty. In this way, the work may help to evaluate the propriety of the current extensive use of F in dentistry, i.e., affirm its safety or intimate that F has physiopathological effects on the pineal gland.

CHAPTER 3 - Methodologies

3.1 Determination of the Fluoride Content of Human Pineal Gland, Bone and Muscle

3.1.1 Collection of Human Pineal, Bone and Muscle Samples

The pineal glands and corresponding bone and muscle samples were dissected from 11 aged cadavers (7 females and 4 males) in the Anatomy Department, UCL. The mean age was 82 years ranging from 70 to 100 years. The subjects had been preserved since the time of death by intravenous injections of a formalin preparation.

Table 3.1 Age, gender, and cause of death of the subjects

Subject	Gender	Age (years)	Cause of death
1	M	79	myocardial infarction
2	F	78	congestive cardiac failure
3	M	78	acute asthma
4	M	85	bronchial pneumonia
5	F	88	ischaemic heart disease
10	F	100	acute renal failure
12	M	81	ischaemic heart disease
13	F	83	pulmonary oedema
14	F	70	cerebral infarction
15	F	75	cerebral infarction
16	F	85	myocardium vascular disease

3.1.2 Analysis of Fluoride

There were two basic stages in the determination of the [F] of all the samples: (a) the preliminary separation of F from the samples by diffusion; (b) the subsequent direct measurement of the [F] of the diffusates using the F-electrode.

3.1.2 (a) Separation of Fluoride

Fluoride in the samples was isolated using the acid diffusion/hexamethyldisiloxane (HMDS) method described by Taves (1968) and modified by Whitford and Reynolds (1979). Taves discovered that F was rapidly released when he applied silicone grease (as opposed to other sealants) to the diffusion dishes used for separating F from biological samples. This implied that silicone grease contained some volatile fraction that somehow participated in the quantitative diffusion of F. Based on this discovery, Taves simply added hexamethyldisiloxane, (HMDS), the simplest silicone, to the acid used for the diffusion of F and noted that: 98% of added ^{18}F could be recovered from bone, urine and serum samples in one to six hours; 800 nmoles of HMDS brought about the diffusion of 4500 nmoles F from bone in one hour; and F could be diffused from samples at room temperature in less than a tenth of the time taken by any previous diffusion method. Diffusion at room temperature, rather than at 55°-60°C, decreases the likelihood that the trapping solution will be contaminated by other volatile components. It also means that disposable plastic dishes can be used (Taves, 1968).

Hexamethyldisiloxane presumably increases the rate of diffusion of F from acidified solutions by the formation of trimethylfluorosilane (TMFS):-

$$H^+ + (CH_3)_3SiOSi(CH_3)_3 \text{ —— } [(CH_3)_3Si]^+ + (CH_3)_3SiOH$$

HMDS — trimethylsilanol

A F ion from the sample or standard solution bonds with the silane radical to form trimethylfluorosilane (TMFS):

$$F^- + [(CH_3)_3Si]^+ \text{ —— } (CH_3)_3SiF$$

trimethylfluorosilane

TMFS, a highly volatile (b.pt. 16.4°C) and hydrophobic compound, facilitates the rapid release of F from an acid solution. When TMFS diffuses into the alkaline absorbing solution, F is exchanged for OH^- and trimethylsilanol is reformed. This occurs by mass action because the OH and F ions have similar affinities for the silane radical:

$$(CH_3)_3SiF + OH^- \text{ —— } (CH_3)_3SiOH + F^-$$

Two molecules of trimethylsilanol recondense to form the parent HMDS, which returns to the acidic solution and the cycle is repeated.

Whitford and Reynolds (1979) simplified the method by placing the alkaline trapping solution as five drops on the underside of the top half of the diffusion dish. See figure 3.2.

3.1.2 (b) Determination of F-Concentration by Potentiometry

The F-concentrations of the diffusates were directly measured using a F ion-specific combination electrode (Orion Research, model 96-09). The sensing element is a single crystal of lanthanum fluoride (doped with europium II to increase its conductivity) which allows free movement of F within the immobile framework of lanthanum ions. It separates the sample and the internal filling solution (Orion, No. 900001). The built-in reference electrode is immersed in the internal filling solution. When the crystal is in contact with a solution containing F, a electrochemical gradient develops across the crystal and gives rise to a potential difference. The response is completely specific to F as no other ions can penetrate into the crystal. The potential difference, which depends upon the level of free F in solution, is measured against the constant built-in reference potential with a pH/mV meter. The measured potential is described by the Nernst equation:

$$E = E_0 + S \log (A)$$

where E is the measured electrode potential; E_0 is the reference potential (a constant); S is the electrode slope (about 57 mV per decade); A is the F-activity level in solution.

The electrode shows a Nernstian response from about 10 M (a saturated F solution) down to 10^{-5} M or 10^{-6} M F-activity and so can be used to determine F-activity over a wide range. It is limited in extremely dilute F-solutions by the sparing but finite

solubility of lanthanum fluoride crystal. The F-activity is directly proportional to the F-concentration if the background ionic strength is high and constant relative to the sensed F-concentration. Therefore, the measured electrode potential varies as the logarithm of the F-concentration.

Electrode potentials of standard F-solutions are measured and plotted on the linear axis against their concentrations on the log axis to produce a calibration curve.

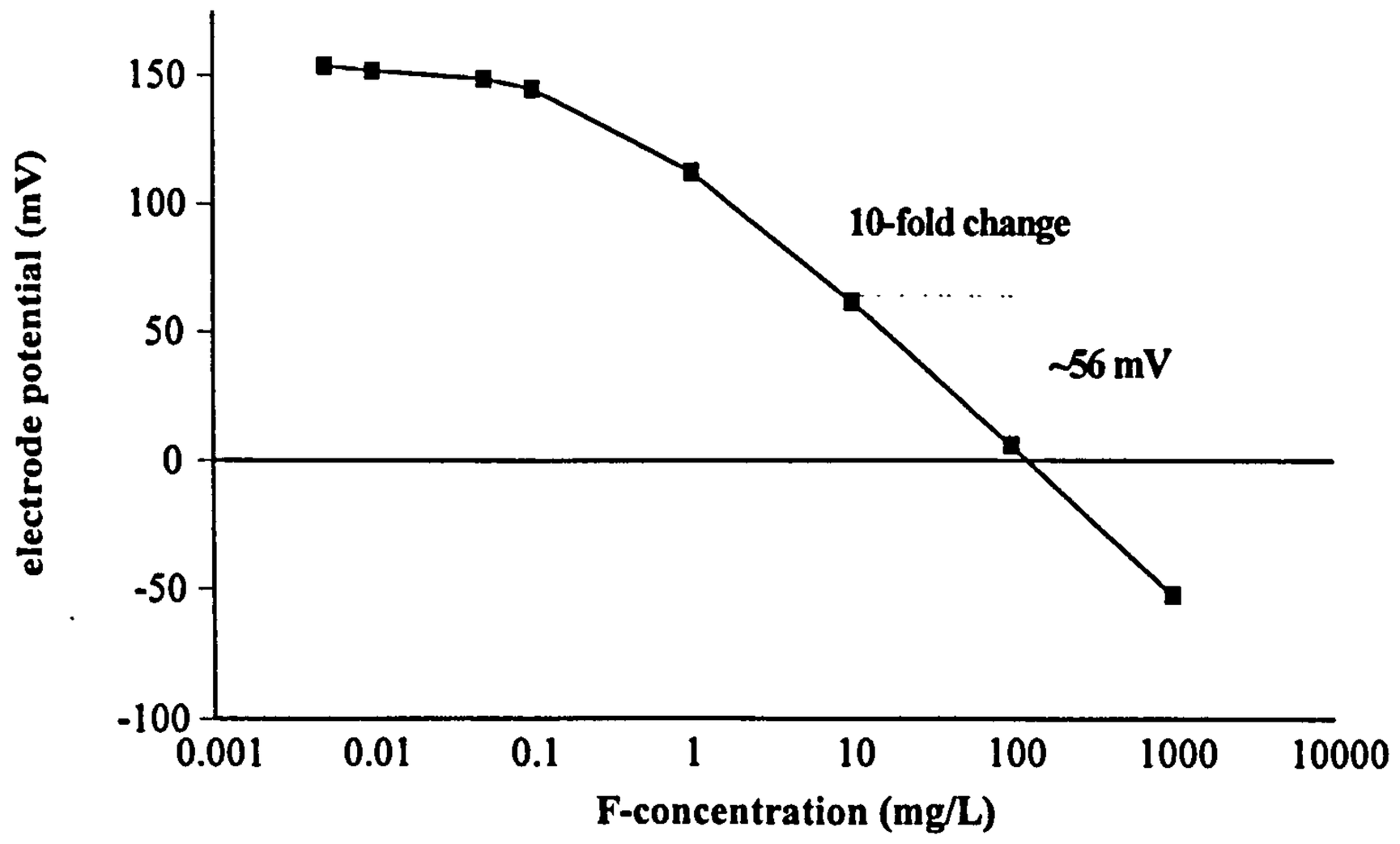

Fig. 3.1 Typical standard curve obtained with the F ion-specific electrode (Orion, model 96-09)

There is an essentially linear region for concentrations greater than 0.1 mg F/L with a slope of about 54-60 mV per decade. When the F-concentration falls below 0.1 mg/L, (1 x 10^{-5} M), the line begins to curve and lose reproducibility. A typical calibration curve for the F-electrode used in this research project is shown in figure 3.1. The limit of sensitivity was about 0.1 mg F/L (1 x 10^{-5} M).

The potential developed by the F-electrode is affected by changes in pH, ionic strength and temperature. In solutions with pH > 8, the F-electrode is sensitive to OH ions because they have a similar charge and ionic radii as F. In solutions with pH < 3.5, undissociated hydrofluoric acid is formed (pK_a HF 3.45). This results in an under-estimation of the [F] because the electrode is not sensitive to HF. Buffers of high ionic strength are added so that any variation in the ionic strength of the sample or standard solution has only a negligible effect on the buffered system. In addition, buffers maintain the pH at about 5 which eliminates interference from OH^- and ensures that the fluoride in solution remains ionic.

However, when a buffer is added to a very dilute F solution, the F is diluted to even lower concentrations: beyond the limit of detection of the electrode. To overcome this problem, F in a very dilute solution has to be quantitatively transferred to a solution with a smaller volume so that the F-concentration is less dilute and above the limit of detection of the electrode.

3.1.3 Experimental Procedure for Analysis of Fluoride

3.1.3 (a) Apparatus and Reagents

F ion-specific combination electrode (Orion, model 96-06; Orion Research UK, Forest Row, Sussex, UK)

pH/mV/ISE meter (Orion, model SA 720)

non-wettable polypropylene diffusion dishes (Griffiths and Neilsen, Billingshurst, Sussex, UK)

hexamethyldisiloxane, (HMDS), Dow Corning 200 fluid, 0.65 centistokes (Aldrich Chemical Co Ltd, New Road, Gillingham, Dorset, UK)

0.1 M NaF, Fluoride Activity Standard (Orion, No. 940906)

$HClO_4$, (Aristar), NaF, (Aristar), glacial acetic acid, (Aristar), H_2SO_4, NaOH, (Analar), are all available from Aldrich Chemical Co Ltd.

Double-distilled water from all-glass still

Solutions for fluoride analysis

0.05 M NaOH stored in a soda-lime desiccator

0.2 M acetic acid

6 M H_2SO_4/HMDS was prepared by adding 84 ml conc H_2SO_4 slowly to 300 ml d-H_20 and, after cooling, making up to 500 ml with d-H_20. The 6 M H_2SO_4 was cooled in the refrigerator for one hour and the volume was readjusted to 500 ml. The acid was then poured into a 1000 ml separatory-funnel and 12-15 ml HMDS was added. The separatory funnel was shaken for five minutes; during which time, the stopper was removed periodically to allow the escape of volatile HMDS. The stopper to the funnel was not sealed overnight so that excess HMDS could escape.

3 M H_2SO_4/HMDS was prepared by mixing equal parts of 6 M H_2SO_4/HMDS and d-H_20.

2 M $HClO_4$ was prepared by adding 10 ml conc $HClO_4$ (60% w/w) to about 30 ml d-H_20 and making up to 50 ml.

Preparation of NaF standards

(i) Stock F standards

Stock F standards were prepared using serial dilutions of 0.1 M NaF standard (Orion) with d-H_20 as follows:

NaF Standard	Standard	Water
10 mM F	50 ml 100 mM NaF	up to 500 ml
1.0 mM F	50 ml 10 mM NaF	"
0.5 mM F	25 ml 10 mM NaF	"
0.1 mM F	50 ml 1 mM NaF	"
0.025 mM F	25 ml 0.5 mM NaF	"
0.01 mM F	50 ml 0.1 mM NaF	"

A stock standard solution of 1.0 M F was made by drying about 1.5 g of NaF at 110°C for 1 hour, followed by cooling in a desiccator for 30 minutes. 1.05 g of the dried NaF was dissolved in about 15 ml d-H_2O and made up to 25 ml with d-H_2O mixing thoroughly with a magnetic stirrer.

All stock F-standards were stored in polypropylene screw-top containers because F tends to adsorb on glass.

(ii) Non-diffused F-standards

Non-diffused F-standards were prepared by mixing 1 ml 0.05 M NaOH with 400 µl 0.2 M acetic acid in 5 ml polypropylene beakers and adding stock NaF standard to make a total volume of 1.5 ml as follows:-

Standard	Volume of stock NaF standards
0.5 nmoles F	100 µl 0.1 mM NaF
1.0 nmoles F	20 µl 1 mM NaF + 80 µl H_20
2.5 nmoles F	100 µl 0.5 mM NaF
5.0 nmoles F	100 µl 1.0 mM NaF
10.0 nmoles F	20 µl 10 mM NaF + 80 µl H_20
25 nmoles F	100 µl 5.0 mM NaF
50 nmoles F	100 µl 10.0 mM NaF
100 nmoles F	20 µl 100 mM NaF + 80 µl H_20
500 nmoles F	100 µl 100.0 mM NaF
1000 nmoles F	20 µl 1 M NaF + 80 µl H_20

(iii) Pre-diffused and diffused F-standards

Pre-diffused and diffused F-standards were prepared by pipetting the following volumes and concentrations of stock NaF standards into the petri dishes:

Standard F	Volume	Stock NaF Standards
0.5 nmoles F	50 µl	0.01 mM
1 nmoles F	100 µl	0.01 mM
2.5 nmoles F	100 µl	0.025 mM
5 nmoles F	50 µl	0.1 mM
10 nmoles F	100 µl	0.1 mM
25 nmoles F	50 µl	0.5 mM
50 nmoles F	50 µl	1.0 mM
100 nmoles F	100 µl	1.0 mM
500 nmoles F	50 µl	10 mM
1000 nmoles F	100 µl	10 mM
2500 nmoles F	250 µl	10 mM
5000 nmoles F	50 µl	100 mM
10 000 nmoles F	100 µl	100 mM
20 000 nmoles F	200 µl	100 mM

3.1.3 (b) Methodology

The petri dishes were labelled and set up: a) in triplicate for the diffused and pre-diffused standards; b) as single or as replicates of six depending upon the sample. See Section 3.1.4. A two mm hole was melted in each lid with a soldering iron. The inside periphery of the lid (where it makes contact with the dish) was ringed with Vaseline. The base trap, typically 50 µl 0.05 M NaOH, was pipetted as five drops on the inside of the top lid. Two ml d-H_20 was placed in the bottom of each dish. Known volumes of liquid samples (bone solutions or F-standards) or known weights of solid samples (pineal tissue or soft tissue) were added to the d-H_20. The lids were placed on the dishes and turned to get a good seal. Two ml of 3 M H_2SO_4/HMDS was injected through the hole in the lid of the already closed dish; the hole was immediately sealed with Vaseline and a small strip of parafilm.

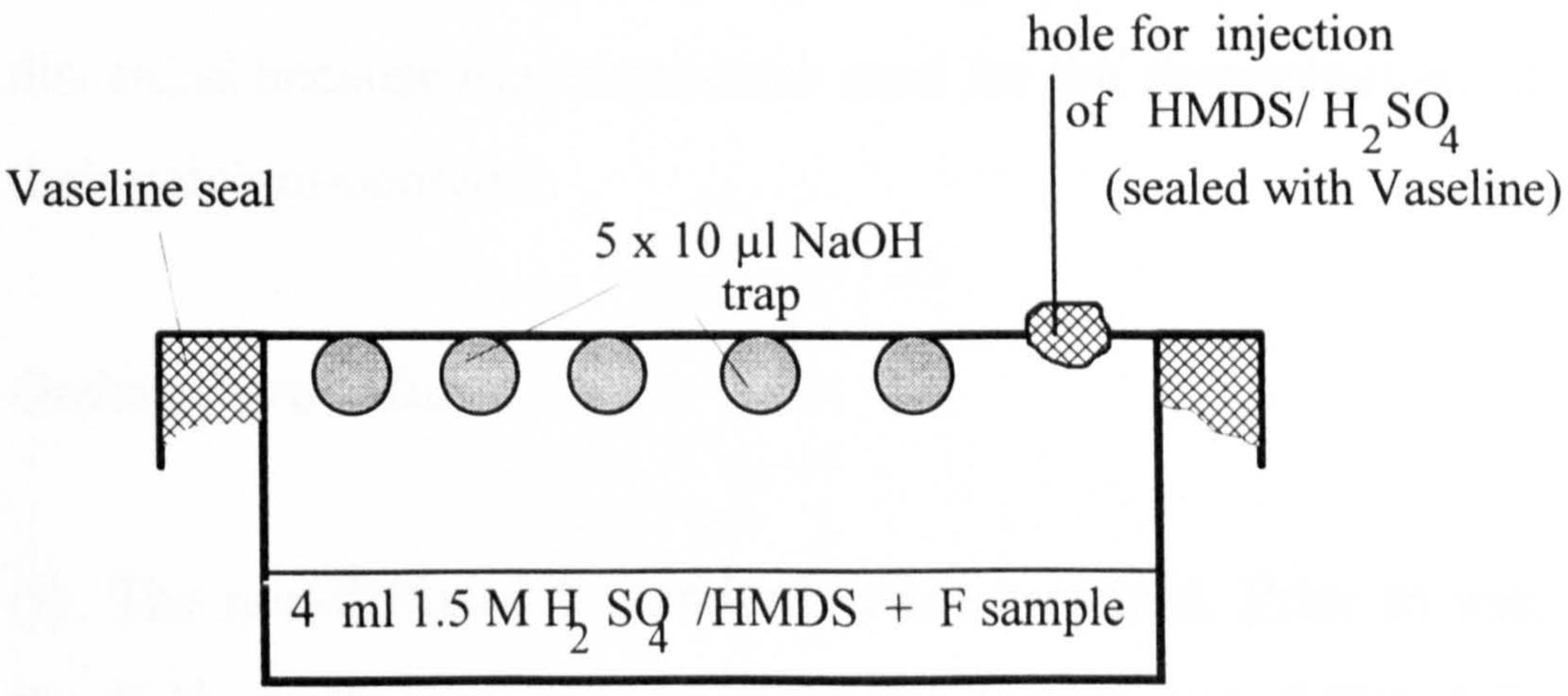

Fig. 3.2 A diagram to show the features of the HMDS-facilitated method for the isolation of ionic and acid-ionizable fluoride. (Reproduced from Whitford, 1996, with permission).

The petri dishes were placed on an automatic horizontal shaker running at 45 cpm for 18 hours.

The next morning, the lids of each dish were removed in turn. 25 µl 0.2 M acetic acid was added to the five 10 µl drops of sodium hydroxide trap in the lid and the two solutions were mixed together using a 75 µl Gilson pipette. The final volume was carefully adjusted to 75 µl with d-H_2O (to compensate for any evaporation that may have occurred during the night) and the acetate buffered F-solution (pH ~ 4.8) was returned into the lid of the petri dish. The tip of the F-electrode was immersed in the acetate-buffered F-solution in the petri lid for the measurement of the F-concentration. The [F] of the unknown samples were determined by interpolation from the calibration curve obtained by plotting log [F] *vs*. electrode potential (mV) for similarly treated standards.

N.B. The acid digests from the pineal gland samples were not discarded because they were later used for the determination of their calcium-contents.

Order of procedure

(i) The non-diffused F-standards were prepared. Prior to use, the F-electrode was immersed in the lowest non-diffused F-standard for 15 minutes in order to 'warm it-up'.

(ii) The mV values for first triplicate of F-standards, (both non-diffused and diffused), were read using the F-electrode.

(iii) Half the samples were prepared and their mV values recorded; followed by the second triplicate of F-standards (non-diffused and diffused).

(iv) The remainder of the samples were prepared and read; followed by the third triplicate of F-standards.

Using this procedure, the recoveries of F can be checked by comparing the mV values from the non-diffused and the diffused F-standards. They should read within 1 to 2 mV. The electrode tip was rinsed with distilled water and blotted dry with paper tissues between each reading. The time response of the electrode (time required to reach 99% of the stable potential reading) varies from several seconds in concentrated solutions to several minutes in solutions which are near the limit of detection of the electrode. Therefore, adequate time was allowed for the mV readings to become stable before recording them.

3.1.3 (c) Limitations of the HMDS Method

(i) Strength The alkaline trap can only take up a finite amount of F because its maximal trapping capacity depends upon the number of OH^- present, e.g., 50 µl 0.05 M NaOH can trap 2.5 µmoles F. If samples contain more than 2.5 µmol F, the strength of the trap has to be increased.

(ii) Pre-diffusion Carbon dioxide is given off by some samples when treated with acids. This combines with OH^- in the trap and reduces its trapping capacity. To avoid this problem, 6 M

H_2SO_4 (typically about 20% of the sample volume) is added to the samples whilst they are still in the unsealed petri dishes so that any carbon dioxide escapes and does not neutralize the trap. After a period of 10 to 15 minutes, 3 M H_2SO_4/HMDS is added in the usual way. The pre-diffused F-standards are treated similarly.

3.1.4 Modifications of Method for Use with Various Tissues

3.1.4 (a) Human Pineal Glands

The pineal glands were blotted with paper tissue to remove excess moisture, weighed to the nearest mg, homogenized in one ml d-H_2O using an agate pestle and mortar and sonified for 10 minutes. A pineal gland, (No. 5), was chosen at random for the determination of its F-concentration. It was placed in one petri dish and the trap and reagents were the typical concentrations. The pineal and pre-diffused F-standards were treated with 200 µl 6 M H_2SO_4 for 10 minutes to drive off CO_2. The standards contained 10, 100, and 1000 nmoles F. Diffusion time was three days. The mV value obtained for No. 5 was outside the highest F-standard on the calibration curve.

The following changes were made to the protocol. A homogenized pineal gland, (No. 12), was divided into two equal parts and the two portions analysed separately. The pre-diffused F-standards and the two portions of homogenized pineal gland were pre-treated with 200 µl 6 M H_2SO_4 for 10 minutes. The concentration and volume of the base trap were

increased to 0.5 M NaOH and 100 µl respectively; the strength of the acetate buffer was increased to 50 µl 2 M acetic acid and the volume of the analysed solution was adjusted to 150 µl with d-H_2O. The standards contained 1000, 2500, and 5000 nmoles F. The diffusion time was three days.

This modified protocol was used for the remaining pineal glands except that the F-standards contained 1000, 10 000, and 20 000 nmoles F.

3.1.4 (b) Bone (Human and Gerbil)

Prior to F-separation, the bone samples were pre-treated to destroy all organic matter. They were cleaned of any adherent soft tissue with a razor blade, dried in an oven at 110°C overnight, and ashed (in porcelain crucibles with no fixatives) in a muffle furnace at 550-600°C overnight. The bone ash was pulverized into a fine powder using an agate pestle and mortar. In order to avoid the inevitable dispersion of the bone ash (due to the static charge in the petri dishes) during transfer from the balance to the petri dishes, bone solutions, rather than bone ash, were used. Bone solutions were made by dissolving known weights of bone ash in 3 ml 2 M $HClO_4$. Aliquots of bone solutions were analysed for F in replicates of six. No pre-diffusion of the samples or standards was necessary; the standards contained 10, 50, 100 and 500 nmoles F; diffusion time was 18 hours. Mean bone [F] values and coefficients of variation between the replicates were calculated.

3.1.4 (c) Muscle

Approximately 100 mg muscle tissue was weighed to the nearest mg, homogenized in one ml water with an agate pestle and mortar and sonified for 10 minutes. Each sample was divided into two parts which were analysed separately. The samples and diffused standards were treated with 200 µl 6 M H_2SO_4 for 10 minutes to drive off CO_2. The standards contained 5, 50, and 500 nmoles F; diffusion time was three days.

See Appendix A for laboratory results.

3.2 Determination of the Concentration of Ionized Calcium in Human Pineal Glands Using Flame Atomic Absorption Spectrometry

Quantitative analysis of calcium using emission spectroscopy depends upon the fact that the intensity (quantity of light) emitted in a given spectrum line is proportional to the number of atoms vaporised and excited. A solution of a calcium compound is sprayed into a flame of acetylene and air. A beam of radiation from a calcium-containing source passes through the flame (wavelength 422.7 nm, identical to that emitted by the energised calcium atoms). A certain fraction of the light intensity is absorbed by atoms which are in their ground electronic state. The quantity of radiation absorbed is directly proportional to the concentration of atoms present.

3.2.1 Experimental Procedure

3.2.1 (a) Digestion of Pineal Samples

The acid digests, which remained in the petri dishes following the separation of F from the human pineals, were placed in clean glass tubes and 1 ml concentrated HNO_3. was added. Using the Autostep Controller 1012, the tubes were heated slowly to 50°C and maintained at 50°C for 30 minutes in a fume cupboard. The procedure was repeated using 1 ml 60% $HClO_4$ (Aristar). Two ml d-H_2O was added to each tube. The volumes of the solutions were measured.

3.2.1 (b) Apparatus and Reagents

The Atomic Absorption Spectrometer, IL 353, was set up for calcium:-

Lamp current	7 mA
Wavelength	422.7 nm
Slit width	320 µm
P.M. Voltage	530 V
Chart range	20 mV
Chart speed	20 mm/min

Reverse osmosis/deionised water (RO/DI)

Calcium nitrate standard solution 1 mg/ml, (SpectrosoL, BDH Prod No 14136 4J)

Lanthanum chloride, (SpectrosoL, BDH Prod No 14041 5W)
Diluent, (0.1% v/v acidified $LaCl_3$ solution). 10 ml $LaCl_3$ solution was diluted to 1 litre using RO/DI water. 10 ml conc HCl was added and the solution was mixed.

3.2.1 (c) Methodology

Preparation of Calcium Standards: 25 mM $Ca(NO_3)_2$ standard solution (1 mg Ca/ml) was diluted into plastic 'Teklab' tubes to make the following calcium standards:

Calcium conc mmol/l	Stock ml	RO/DI water ml
1.5	0.6	9.4
2.0	0.8	9.2
2.5	1.0	9.0
3.0	1.2	8.8
3.5	1.4	8.6
4.0	1.6	8.4
5.0	2.0	8.0

The calcium standards, quality controls (QCs), blanks and the digested pineal samples were diluted 1 + 50 (50 µl of standard with 2.5 ml diluent) using the Hamilton Diluter and then vortexed.

The calcium standards, samples, QCs and blanks were aspirated through the Atomic Absorption Spectrometer, IL 353. The correct dilution of the samples was found which gave an absorption reading within the range of the standards. Most samples were diluted 1:200 with lanthanum chloride; No. 3 needed further dilution to 1:400. Absorbance was recorded using a PM 8251 single pen recorder.

A line was drawn through the bottom of the standards and the peak heights were measured from this line. Peak height was plotted against standard concentration to produce a calibration

curve. The concentrations of ionized calcium in the samples were determined from the calibration curve.

3.3 Animal Maintenance

Eight monogamous breeding pairs of Mongolian gerbils (Bantin and Kingman Ltd, Grimston, Aldbrough, Hull, UK) provided all the animals used in this study. Upon arrival at the Animal Unit, the breeding pairs were housed in a separate room away from the other animals. The temperature was 22 ± 2°C. The lighting was provided by Vitalight (Full Spectrum Lighting Ltd, Sunbury-on-Thames, Middlesex, UK). The LD: 12 12 cycle was controlled automatically with lights on at 0700 and lights off at 1900 daily. The breeding pairs were housed in standard rat cages with a floor area of 60 x 36 x 20 cm, bedded with wood shavings. Hay was provided for nesting. They had free access to commercial rodent food (LAB animal diet, No. 1, from Lillico, UK) and double distilled water.

Cages were checked daily for birth of pups and dates of birth were recorded. Litters were randomly assigned to either the high-F (HF) or low-F (LF) group. HF pups were given a daily F-solution (orally by Gilson pipette) from day 1 until they were weaned onto a high-F diet at 24 days. (See below). All pups were weaned at 24 days postpartum and separated from their parents. Pups (from different breeding pairs) were assembled into one-sex groups of four per cage for the duration of the experiment under LD: 12 12. Their plastic cages were fitted with open wire-work tops and measured 60 x 36 x 20 cm. Wood shavings were provided for bedding and hay for nesting. The appropriate rodent foods and distilled water

were available ad libitum. The litters were weighed weekly; and individuals identified by ear clippings. The breeding pairs, sexually mature and immature gerbils of both sexes and from both groups were maintained in the same room.

Calculation of the daily F-dose

From day 1, the HF pups received 2.3 µg F/g BW/day, Monday to Friday. The approximate body weight in relation to age was:

birth to 7 days	3 g
7-14 days	9 g
14 -24 days (weaning)	15 g

Therefore, the HF pups received 7 (3 x 2.3); 21 (9 x 2.3); and 35 (15 x 2.3) µg F/day in weeks 1, 2 and 3 respectively. The total daily F-dose was divided into three; each third of the dose was present in 10 µl (i.e., total daily F-dose was in three 10 µl drops). At 1000, 1330 and 1700, the pups received one 10 µl drop orally using a Gilson pipette.

Therefore, the pups received a daily F-dose of 7, 21 and 35 µg F in 30 µl in weeks 1, 2 and 3 respectively. Solutions were made up containing 50.8, 152.5 and 254.2 mg NaF in 100 ml water for use in weeks 1, 2 and 3 respectively.

From day 1 until weaning at day 24, HF pups received 10 µl of the appropriate NaF solution orally by Gilson pipette (3 times/day, 5 days/week).

From day 24 and for the duration of the experiment, the HF group received a high-F, pelleted, rodent food. The 'natural ingredient' diet consisted of a mixture of wheat, soya, oats, fish meal, (no bone meal) vitamin mix supplemented with NaF to provide 37 mg F/kg (SDS, Witham, Essex, UK). This F-content was chosen because it was not unrealistically high and compared well to the F-contents of rodent feeds used in previous animal studies. The LF group received the same diet without the added NaF and provided 7 mg F/kg (SDS, Witham, Essex, UK). Both groups had free access to their appropriate feeds and distilled water. Hay was provided for nesting (parts of which they presumably ate).

3.4 Collection of Urine Samples

Urine was collected from individual gerbils housed in metabolic cages as follows:

(i) The Longitudinal Study: urine was collected at 3-hourly intervals over two consecutive days from 24 gerbils (12 females, 12 males) from the HF and LF groups at 7, 9, 11½ and 16 weeks of age: at 0700, 1000, 1300, 1600, 1900, 2200, 0100 and 0400.

(ii) The Additional Study: urine was collected at 24-hourly intervals over two consecutive days from surplus gerbils from the HF and LF groups at various ages (9, 11½, 16 and 28 weeks). These gerbils were born and raised under identical conditions as those used in the longitudinal study. The levels of urinary aMT6s excreted by the additional gerbils were not monitored over puberty. The results were used to supplement data from the longitudinal study and so make the statistical analyses more powerful.

Each gerbil was placed inside a glass, open-ended cylinder resting on a metal grid. Urine fell through the grid onto the sides of the glass funnel and ran into a collecting bottle. Faeces and waste food fell directly through the grid and funnel. See figure 3.3. At the end of each 3-hourly interval, the inside of each glass funnel was rinsed with about 10 ml d-H_20 to ensure the maximal recovery of the urine which had been excreted during that interval. The gerbils received pelleted, stock rodent food (LAB animal diet, No 1, from Lillico) ad libitum during their time in the metaboles because the pellets of their specially prepared food were small and could pass through the grid. Therefore, to maintain the F-regime whilst in the metaboles, the HF group received drinking water containing 50 mg F/L. The LF group received distilled water. During the dark phase, the urine samples were collected under illumination provided by a dim red light. The urine samples were stored at -20°C without any additives. Prior to assay, the urine samples were thawed, centrifuged at 2000 rpm for 10 minutes and the volumes of the supernatant fluids were measured.

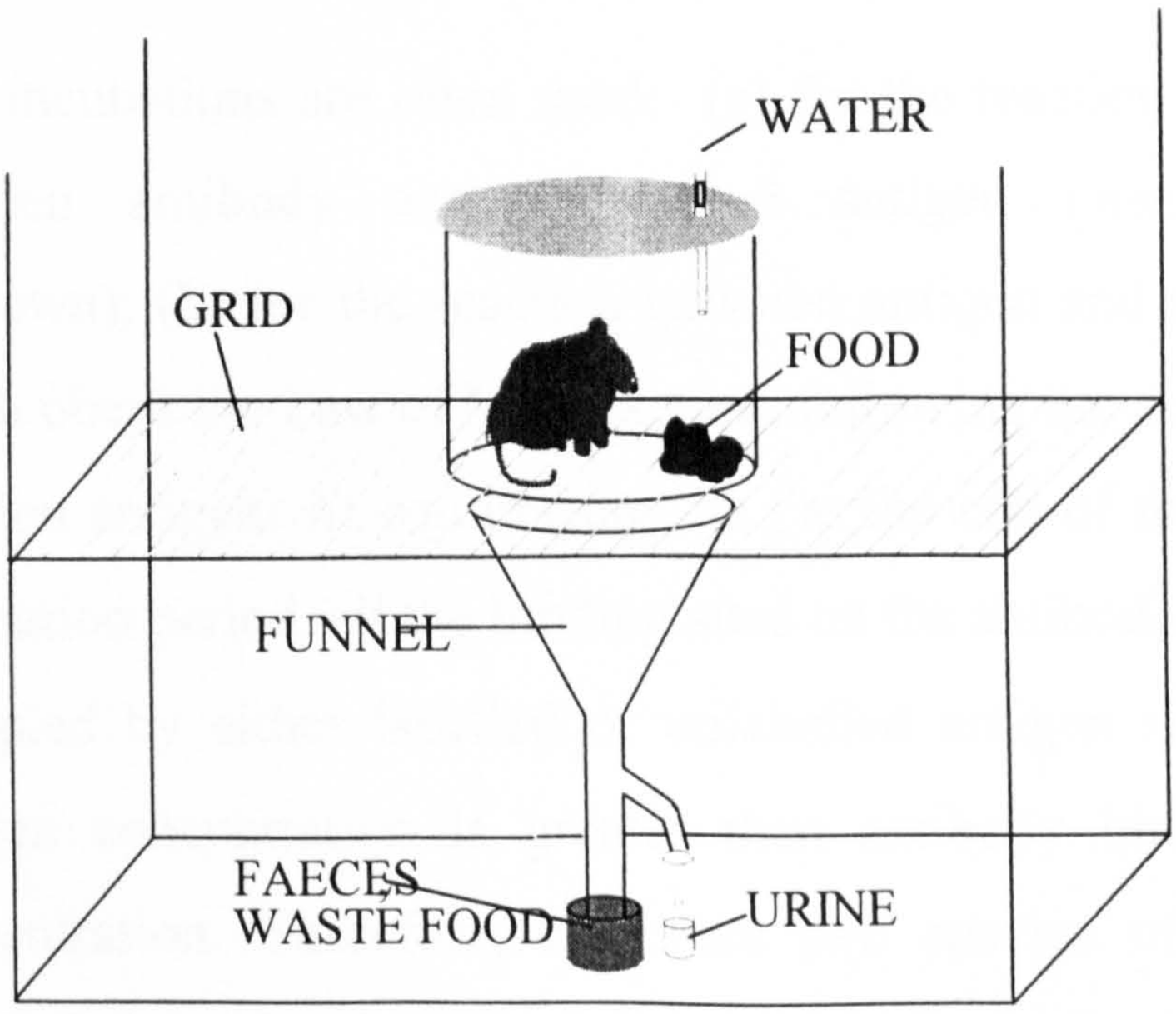

Fig. 3.3 Diagram of metabole used to collect gerbil urine

3.5 Measurement of Urinary Sulphatoxymelatonin Using Radioimmunoassay

3.5.1 Principles of Radioimmunoassay

Radioimmunoassay (RIA) is a highly sensitive, reliable and specific, in vitro method of measuring a substance in biological samples. In theory, it can be applied to any substance to which an antibody can be raised: an enormous range of substances. The technique involves the competition of a constant amount of radiolabelled antigen with a variable amount of antigen present in the unknown sample for a fixed yet limited number of specific antibody binding sites. The radioisotopes labelling the antigens in the RIAs of urinary aMT6s and MT are ^{125}-I (gamma-emitter) and H^3 (ß-emitter) respectively. The antigen is either the pure standard preparation or part of the biological sample under investigation.

Two incubations are often used:- (a) for the reaction to occur between antibody and unlabelled antigen (standard or unknown); (b) for the reaction between antigen and antibody, which obeys the Law of Mass Action, following the addition of labelled antigen. At equilibrium, i.e., at the end of the second incubation period, all the binding sites on the antibodies will be occupied by either labelled or unlabelled antigen since total antigen concentration is greater than antibody binding site concentration. Therefore, there are two species of labelled material: (a) a fraction which is bound to the antibody; (b) the remainder which is free in solution.

At the end of the second incubation period, it is necessary to physically separate the free and antibody-bound antigen from each other without disrupting the established equilibrium. In both RIAs used in this project, this separation was achieved by adding dextran-coated charcoal (DCC) to the assay tubes at the end of the second incubation period. A simple explanation of how DCC achieves this separation is that dextran (MW 80 000) acts as a molecular sieve. The smaller-sized, free labelled antigen is able to penetrate into the charcoal but the larger antigen-antibody complexes are excluded and remain in solution. The final separation is achieved by centrifugation, followed by aspiration or disposal of the supernatants. The distribution of radioactivity between the bound and the free fractions can then be related to the amount of antigen added. The concentration of antigen in the samples is then determined by comparing their radioactivity to that observed in the standard curve where increasing amounts of standard have been added.

In the RIA for urinary aMT6s, the supernatants were discarded and the γ-radiation emitted by ^{125}I-aMT6s in the charcoal pellets (free-fraction) was counted using a crystal scintillation system. In the RIA for pineal MT, the ß-radiation emitted by ^{3}H-MT in the supernatants (bound fraction) was counted in a liquid scintillation medium.

3.5.2 Materials

Water: All water was freshly double-distilled in glass.

Buffer: Tricine buffer (Prod No T-0377, Sigma Chemical Co, Poole, Dorset, UK) was made up at 0.1 M, pH 5.5, with 0.9% sodium chloride (BDH, Poole, Dorset, UK) and 0.1% gelatine (BDH). 17.9 g tricine, 9.0 g NaCl and 1.0 g gelatine were made up to 1 litre with d-H_2O and heated to 50°C for 30 minutes to dissolve the gelatine. It was stored at 4°C and made up weekly.

Antiserum: Anti-aMT6s antiserum, (Batch No 1118/23884, Stockgrand, Guildford, Surrey, UK). 6-sulphatoxymelatonin is a small molecule and does not elicit an immune response by itself. In order to produce antibodies, aMT6s was first conjugated through the side chain to ovalbumin, using the Mannich reaction, a formaldehyde condensation reaction (Franey, 1988). Six Suffolk cross ewes were immunized with aMT6s conjugate emulsified in modified Freund's adjuvant-saline by subcutaneous injection at six sites on the back and legs. Seven months later, the sheep were boosted by injecting with smaller quantities of aMT6s conjugate. They were bled for the collection of antiserum nine days later. The antiserum was supplied freeze-dried and was reconstituted with 1 ml d-H_2O. Nine ml of buffer was added to provide an intermediate dilution of 1:100 and aliquoted into 100 µl portions which were stored at -20°C.

aMT6s standards: Standard aMT6s was synthesized from 6-hydroxymelatonin at the University of Surrey using the method described by Fellenberg *et al*, (1980). Full details of the synthesis are given by Franey (1988). Standard aMT6s (Stockgrand, Guildford, Surrey, UK) containing 500 pg per vial

was reconstituted with 2.5 ml 1:25 charcoal-stripped gerbil urine (see below) to give a working solution of 200 pg/ml.

The standards for the construction of the standard curve were prepared by further dilution of the working solution with 1:25 CSU to give 0, 1, 2, 4, 8, 14, 20, 40, 100 pg/tube as follows:

aMT6s standard 200pg/ml µl	aMT6s-free urine 1:250 dilution µl	aMT6s conc ng/ml
-	500	0
5	495	0.5
10	490	1
20	480	2
40	460	4
70	430	7
100	400	10
200	300	20
500	-	50

Label: ^{125}I-aMT6s (Stockgrand, Guildford, Surrey, UK). The antigen was iodinated directly using Na^{125}I and iodogen as previously described (Aldous and Arendt, 1988). The working solution of ^{125}I-aMT6s was prepared fresh for each assay by diluting the stock solution with tricine buffer to give 10 000 cpm per 100 µl.

Dextran-coated charcoal (DCC): 2 g Norit A charcoal (Prod No. C-5260, Sigma Chemical Co) was suspended in 100 ml tricine buffer and stirred on a magnetic plate for 5 minutes. The suspension was centrifuged for 5 minutes at 1000 rpm at 4°C. The supernatant and the fines around the sides of the vessel were discarded. The sediment was re-suspended in 100 ml

tricine buffer and then stirred with 20 mg Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden) for at least one hour at 4°C. DCC can be used for up to 10 days if stored at 4°C. Before use, DCC was stirred for 30 minutes at 4°C.

Charcoal-stripped urine (CSU) (aMT6s-free urine): Seven adult male gerbils (breeders) were placed in metabolic cages (figure 3.3) during the daytime for the collection of urine. The urines were pooled to give a total volume of 100 ml. Charcoal was de-fined as follows:- 6 g activated charcoal was washed with d-H_2O and centrifuged at 1000 rpm for five minutes. The supernatant was discarded along with any fines around the sides of the container. This procedure was repeated three times. The charcoal was washed with d-H_2O and left to settle and the supernatant was discarded. This was repeated four times. The charcoal was then filtered, washed with acetone, and left to dry at 50°C until there was no smell of acetone. 5 g of de-fined charcoal was added to 100 ml of pooled gerbil urine and was stirred overnight at 4°C. The urine was centrifuged at 3000 rpm for 15 minutes and filtered through Seitz filters (6 cm HP/EF, circular, from H Erben Ltd, Hadleigh, Ipswich, Suffolk, UK) under low vacuum (water pump) until clear. The charcoal stripped urine (CSU) was divided into 500 µl aliquots which were stored frozen at -20°C.

Quality controls (QCs): Urine was collected from several gerbils to provide three pools of urine containing low, medium and high levels of aMT6s: approximately 3, 12 and 24 ng/ml

aMT6s. These spanned the analytical range of the RIA for aMT6s. The pools were aliquoted and stored frozen at -20°C.

3.5.3 Validation of the Assay for Use with Gerbil Urine

3.5.3 (a) Assessment of the appropriate antiserum dilution

The antiserum dilution appropriate to the label or to get about 70% maximum binding was determined using antiserum dilution curves. aMT6s antiserum, (1118/23884), with an intermediate dilution of 1:100, was serially diluted with tricine buffer. Four standard curves for urinary aMT6s were set up using 200 µl antiserum at 1:10 000, 1:20 000, 1:30 000 and 1:40 000 dilutions. B/T% was plotted against concentration of aMT6s (0-50 ng/ml urine) on a semi-logarithmic scale. See Table F.1 in Appendix.

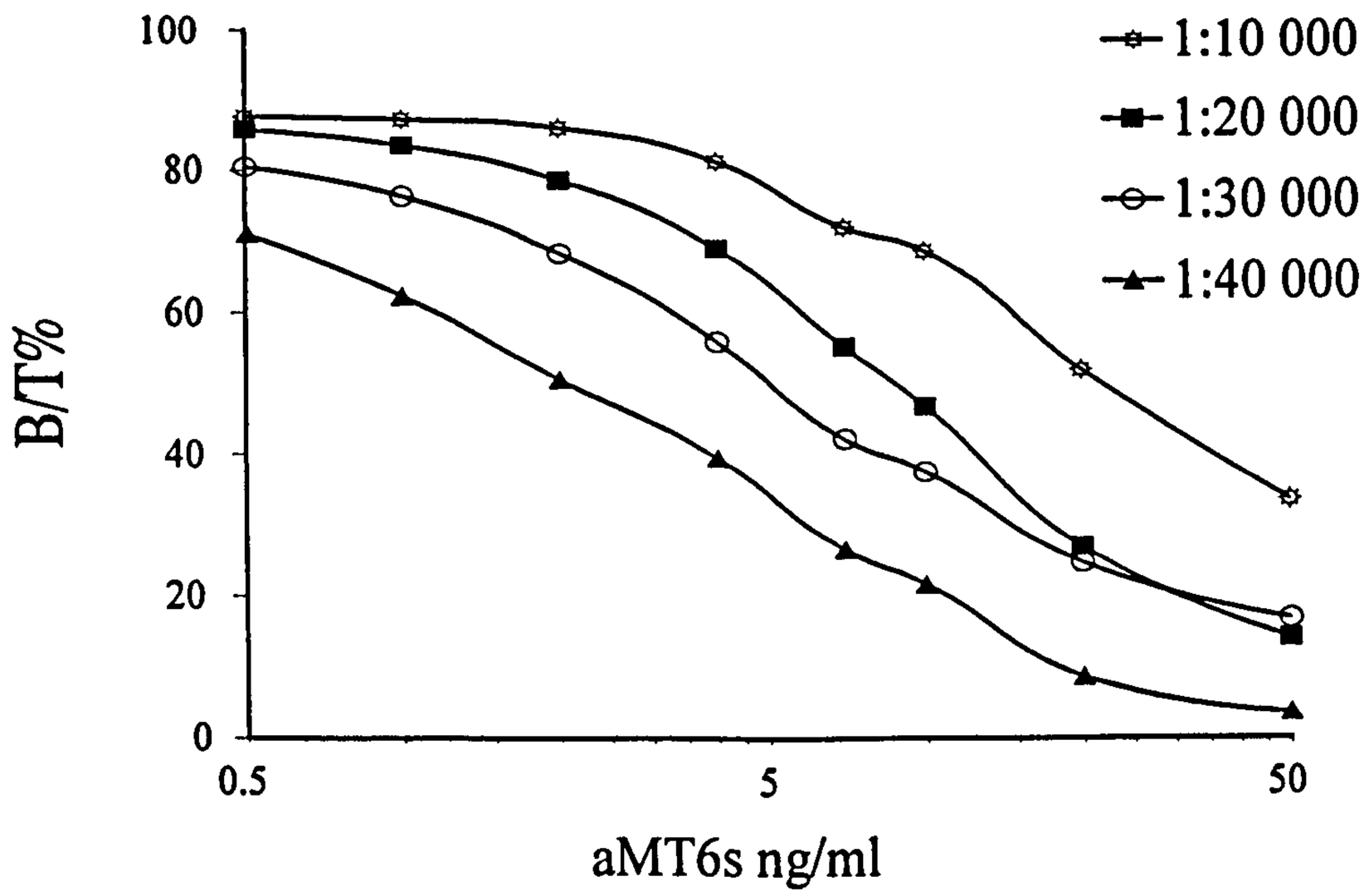

Fig. 3.4 Antiserum dilution curves for urinary aMT6s

From these results it was decided that the optimum dilution of the antiserum would be 1:40 000, i.e., 100 µl of antiserum 1:100 dilution made up to 40 ml with tricine buffer.

3.5.3 (b) Cross-reactivity

Most antisera cross-react to a greater or lesser extent with closely related substances and this interferes with the antigen-antibody reaction. Therefore, the antiserum is characterized for specificity towards molecules which have a similar amino acid sequence as the antigen, e.g., analogues of the antigen, metabolites, breakdown products, etc.

Table 3.2 Cross-reactivity data for antiserum 1118/23884

Compound	pg necessary for 50% displacement of ^{125}I-aMT6s	% cross-reactivity
aMT6s	26	100.0
5-sulphatoxy-*N*-acetylserotonin	1320	2.0
5-glucuronide-*N*-acetylserotonin	1920	1.4
6-glucuronide melatonin	4800	0.5
6-hydroxymelatonin	> 20 000	> 0.11
melatonin	> 20 000	> 0.11
5-methoxyindole acetic acid	> 20 000	> 0.11
5-hydroxyindole acetic acid	> 20 000	> 0.11
N-Acetyltryptamine	> 20 000	> 0.11
N-Acetylserotonin	> 20 000	> 0.11
N- Acetyltryptophan	> 20 000	> 0.11
5-hydroxytryptophan	> 20 000	> 0.11
N-methyltryptamine	> 20 000	> 0.11
5-methoxytryptamine	> 20 000	> 0.11
5-methoxytryptophan	> 20 000	> 0.11
5-hydroxytryptamine	> 20 000	> 0.11
5-methoxytryptaphol	> 20 000	> 0.11
5-hydroxytryptaphol	> 20 000	> 0.11
Tryptophan	> 2 000 000	> 1.1×10^{-3}

Reproduced from Aldous and Arendt (1988)

The specificity of the anti-aMT6s antiserum, (Batch No 1118/23884), was assessed by comparing the ability of several indoles and indole metabolites to displace 50% of maximum antibody-bound ^{125}I-aMT6s under the conditions routinely employed in the assay.

Table 3.2 shows that the percentage cross-reactivity with all the tested compounds was negligible and so they would not compete with aMT6s in the assay. Only 5-sulphatoxy-*N*-acetylserotonin, 5-glucuronide-*N*-acetylserotonin and 6-glucuronide melatonin had low cross-reactivities but as they are present in such small amounts in urine, the antiserum was considered to be sufficiently specific for clinical application without pre-assay treatment.

3.5.3 (c) Effect of CSU on the standard curve

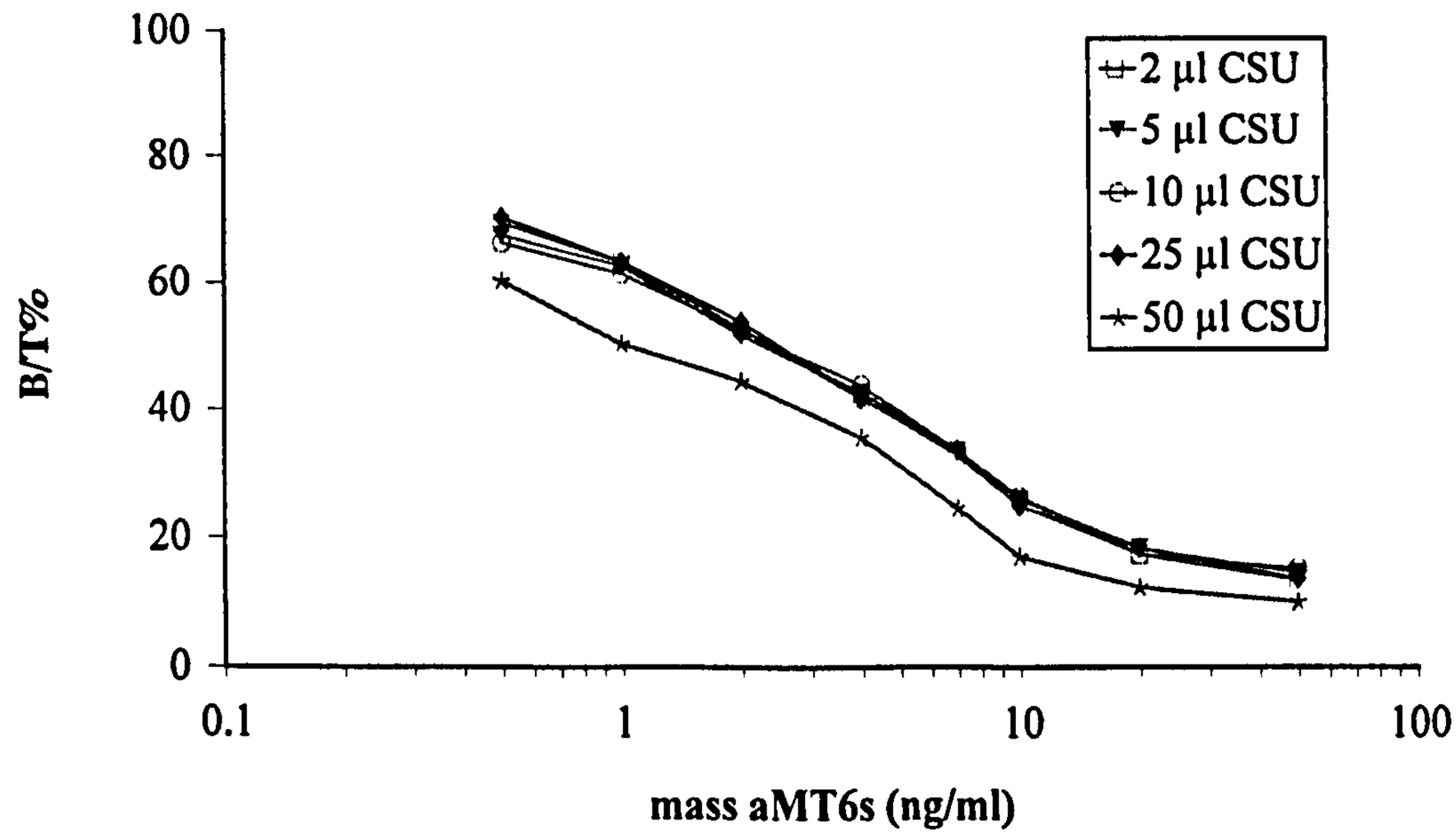

Fig. 3.5 The effects of increasing amounts of gerbil charcoal stripped urine on the standard curve for urinary aMT6s

The addition of increasing volumes of gerbil CSU (2-25 µl) had no effect on the standard curve for aMT6s.

3.5.3 (d) Parallelism

To determine whether the assay was parallel, ten samples of gerbil urine were diluted 1:2 and 1:4 in gerbil CSU and assayed. The results (Table 3.3) show that the samples diluted parallel.

Table 3.3 Serial dilution of ten urine samples with CSU to show parallelism of urinary aMT6s values

	urinary aMT6s (ng/ml)		
Sample	initial concentration	1 : 2 CSU	1 : 4 CSU
1	55.4	30.6	16.2
2	50.6	27.3	13.1
3	32.5	17.1	8.6
4	21.5	10.3	6.1
5	15.4	8.2	4.1
6	42.8	20.6	11.5
7	11.5	5.5	2.7
8	22.6	12.5	5.2
9	32.7	15.3	7.6
10	37.6	15.8	9.2

3.5.3 (e) Sensitivity

The sensitivity of the assay, defined as the least amount distinguishable from zero at the 95% confidence level, was 0.2 ng aMT6s/tube. See Tables F.2(i)-F.2(iii) in Appendix.

3.5.3 (f) Recoveries

Table 3.4 shows that the analytical recovery of aMT6s at three different concentrations was between 98.3 and 102.8% (n =10).

Table 3.4 Quantitative recovery of unlabelled aMT6s

		5 ng/ml aMT6s added		15 ng/ml aMT6s added		30 ng/ml aMT6s added	
sample	0	total	% recovered	total	% recovered	total	% recovered
1	0.5	5.8	106	17.3	112	31.6	104
2	0.8	6.5	114	14.8	93	32.8	107
3	1.5	6.2	94	16.0	97	30.5	97
4	2.4	7.2	96	16.7	95	32.4	100
5	0.4	5.9	110	15.4	100	29.3	96
6	1.7	6.5	96	16.5	99	31.5	99
7	4.6	9.3	94	18.3	91	33.9	98
8	3.7	8.8	102	17.6	93	33.0	98
9	1.2	6.5	106	16.7	103	31.9	102
10	7.2	12.7	110	22.2	100	34.6	91
	mean recovery		102.8		98.3		99.2
	SD		7.4		6.1		4.3

Sections 3.5.3 (c), (d) and (f) were kindly performed by Judy English.

3.5.4 Methodology

The assay procedure was based on that previously described by Aldous and Arendt (1988) with the following modifications. 200 µl of antiserum was used at a dilution of 1:40 000 in buffer. Gerbil CSU was used instead of human CSU.

Dilution of the samples and QCs: The urine samples (which had been diluted with d-H_2O following rinsing of the metaboles) were further diluted with tricine buffer using an automatic diluter. The degree of dilution depended upon the original dilution of the urine collected from the metaboles.

Urine samples collected at 3-h and 24-h intervals were diluted 1:25 and 1:50 respectively. QCs were diluted 1:25. The levels of urinary aMT6s excreted by the 7- and 9-week-old gerbils were assayed on a 24-h basis because there was not enough time to complete the assays on a 3-hourly basis. Before pooling eight 3-h urine samples to give one 24-h sample for each of the 7- and 9- week-old gerbils, 200 µl from each 3-h urine sample was pipetted into Eppendorf tubes and stored at -20°C. The pooled 24-h urine samples were diluted 1:10 with buffer.

Procedure

The assay size was limited to about 160 tubes to avoid assay drift. The volumes required in the assay were:

Sample/standard	500 µl
Antiserum	200 µl
Label	100 µl
Dextran-coated charcoal	100 µl
Total volume	900 µl

The aMT6s standards were made up in 1:25 CSU and 500 µl were dispensed to form the standard curve. (See page 78). Unknown samples, NSBs, and standards were pipetted into LP3 tubes (Luckhams, Burgess Hill, Sussex, UK) in duplicates using Gilson pipettes or repeating Eppendorf microlitre dispensers; zero tubes were prepared in quadruplicate. 200 µl of antiserum at a final dilution of 1: 40 000 was added to all the assay tubes except the total and NSB tubes. The total tubes only contained 100 µl label so that the total radioactivity of the label could be counted. The NSB tubes received 200 µl tricine

buffer instead of 200 µl antiserum to discover whether or not other compounds bind with the antigen. The tubes were vortexed for ten seconds and left to incubate at room temperature for 30 minutes.

After adding 100 µl ^{125}I-aMT6s (10 000 cpm), the tubes were vortexed for ten seconds and left to incubate overnight at 4°C. The bound- and free-aMT6s were separated from each other by the addition of 100 µl DCC, (stirring at 4°C), to all tubes except the totals. After vortexing for ten seconds, the tubes were left to incubate for 15 minutes at 4°C and then centrifuged at 2500 rpm for 15 minutes at 4°C. Immediately after centrifugation, the supernatants were discarded.

The radioactivity in the charcoal pellets, (the free ^{125}I-aMT6s antigen), was counted for 100 seconds in a 12 channel gamma counter (Multigamma, LKB Ltd, Selsdon, Surrey, UK). Standard curves were plotted on B/B_0% (y)/log(x) coordinates.

The aMT6s concentration was determined from the dose-response curve using a 'RIAcalc' program.

The 'RIAcalc' program determines the percentage of total counts (bound or free) and then plots them as a function of the known concentrations of the standards. A curve is fitted through the standard points using a smoothing spline calculation method. This fits a continuous curve to each pair of standard points using the least squares method and it calculates the best fitting curve with a minimum number of turning

points. The concentrations of urinary aMT6s in the unknown samples were determined from the standard curve.

All samples from individual gerbils at any particular age were analysed in the same assay. All analyses were done in duplicate. When the results did not agree within 10%, the sample was repeated and the outlier was discarded. Samples which had aMT6s concentrations greater than 26 ng aMT6s/ml were repeated using a higher dilution. The excretion rate of aMT6s was calculated by multiplying the concentration (ng/ml) with the corresponding urine volume.

3.6 Measurement of Melatonin Content in Gerbil Pineal Using Radioimmunoassay

3.6.1 Collection of the Gerbil Pineals

The subjects were 71 gerbils aged 16 weeks from: the HF group (n = 12 females, 12 males), the LF group (n = 12 females, 12 males) and an additional low-F group (LFNM) which had been used as a pilot study (n = 12 females, 11 males). Groups LF and HF consisted of gerbils whose urinary levels of aMT6s had been monitored at 7, 9, 11½ and 16 weeks; group LFNM consisted of gerbils whose urinary levels of aMT6s had only been determined at 16 weeks of age.

The 16-week-old gerbils from the LFNM, HF and LF groups were sacrificed in July, October and November, 1992,

respectively, the day after they had completed 48 hours in the metaboles for the collection of urine.

Six gerbils, (3 males and 3 females), from each group were sacrificed by cervical dislocation at 6-h intervals over a 24-h period: at 1000 and 1600 (during the light); 2200 (3-h after the onset of darkness) and 0400 (9-h after the onset of darkness). The superficial pineal glands were removed with a minimum of delay, placed in 5 ml Eppendorf tubes containing a drop of d-H_2O and frozen rapidly on solid CO_2. During the dark phase of the LD cycle, the sacrifice of the gerbils and the collection of pineals were performed with the aid of illumination from a dim red light. The pineals were stored at -20°C.

3.6.2 Analytical Procedure

Melatonin concentrations in the gerbil pineals were estimated by means of a sensitive and direct RIA that had been previously validated for other species (Fraser *et al*, 1983; Webley *et al*, 1985). Pineals were homogenized in 1 ml tricine buffer on ice using a glass pestle and mortar, (Glass Unit, University of Surrey). The homogenates were transferred to LP3 tubes and centrifuged at 3500 rpm for 15 minutes at 4°C. The supernatants were aspirated and frozen at -20°C until time of assay.

Estimation of the required volume of pineal extract for RIA

The required volume of pineal supernatant for the RIA was estimated to ensure that the MT concentration lay within the

range of the standard curve for MT: typically 5-250 pg/0.5 ml. This was important because of the limited amount of available sample. The RIA for MT required one ml of sample i.e., 500 µl in each duplicate. Assuming that the gerbil pineal produces about 50 pg MT/day, 200 µl of pineal supernatant contains about 10 pg MT (each pineal was homogenized in 1 ml buffer). Assuming that the gerbil pineals contain higher MT levels during the night than the day, the following volumes of pineal supernatants were used:-

	pineal supernatant	tricine buffer
night-time	200 µl	300 µl
daytime	300 µl	200 µl

3.6.3 Reagents

Water: All water was freshly double-distilled in glass.

Tricine buffer: See page 77

Dextran-coated charcoal (DCC): See page 78

Label: ^{3}H-melatonin (Prod No. TRK-798, from Amersham International, Buckinghamshire, UK) was obtained in 0.25 mCi quantities. An intermediate solution of ^{3}H-melatonin was prepared by diluting 20 µl of stock label to 2 ml with absolute ethanol. The working solution was freshly prepared by diluting the intermediate solution with tricine buffer to give about 4000 cpm in 100 µl.

Antiserum: Sheep anti-melatonin antiserum, (Prod No. G/S/704-8483, from Stockgrand, Guildford, Surrey, UK). The antisera were raised in sheep against *N*-acetyl-5-methoxytryptophan conjugated through the side-chain to

bovine thyroglobulin. It was supplied freeze-dried and was reconstituted with two ml d-H_20 (an intermediate dilution of 1:10) and aliquoted into 50 µl portions and stored at -20°C. One 50 µl aliquot was diluted with 20 ml tricine buffer to give the working solution (initial dilution of 1:4000).

Melatonin standards: Standard melatonin (Prod No. M-5250, from Sigma Ltd). A stock melatonin standard was prepared every three months by dissolving 10 mg melatonin in 0.5 ml absolute ethanol and making up to 10 ml with d-H_20 to give a concentration of 1 mg/ml. The working standard was freshly prepared from this stock solution for each assay.

100 µl of 1 mg/ml standard MT to 100 ml in d-H_20 to give 1 µg/ml MT.
0.5 ml of 1 µg/ml MT solution to 50 ml in d-H_20 to give 10 pg/µl MT.
125 µl of 10 pg/µl MT solution to 2.5 ml in tricine buffer to give 0.5 pg/µl MT.

Further dilution of 0.5 pg/µl MT with tricine buffer provided the standard curve - 0, 2.5, 5, 12.5, 25, 50, 100 and 250 pg MT/0.5 ml as follows:

Melatonin 0.5 pg/µl µl	Tricine buffer µl	Melatonin pg/0.5 ml
0	500	0
5	495	2.5
10	490	5
25	475	12.5
50	450	25
100	400	50
200	300	100
500	0	250

Scintillant fluid: The toluene-based scintillant was prepared by adding 2.5 g 2,5-diphenyloxazole (PPO) (Fisons) and 0.15 g 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene (dimethyl POPOP, from Fisons) to 0.5 L toluene (low-sulphur grade, from Fisons).

3.6.4 Methodology

Except for the zero binding tubes, which were set up in quadruplicate, all standards, samples, totals and NSBs were set up in duplicate with the following volumes of reagents:-

Samples/standards	500 µl
Antiserum	200 µl
Label	100 µl
Dextran-coated charcoal	500 µl
Total volume	1300 µl

500 µl sample (200 µl pineal supernatant and 300 µl buffer, or 300 µl pineal supernatant and 200 µl buffer, depending upon whether the sample was taken during the night-time or day-time respectively), MT standard or QC were added to each duplicate assay tube. 200 µl antiserum was added to all tubes except the totals and NSBs. The tubes were vortexed for 10 seconds and left to incubate for 30 minutes at room temperature. 100 µl ^{3}H-melatonin was added. After vortexing, the tubes were incubated for 18 hours (overnight) at 4°C. 500 µl DCC at 4°C was added to separate antibody-bound MT from free MT. The tubes were vortexed, incubated for 15 minutes at 4°C, and centrifuged at 1500 rpm for 15 minutes at 4°C. 700 µl

aliquots of the supernatants (containing the antibody-bound fraction) were pipetted into vials containing 4 ml scintillant fluid. The vials were thoroughly shaken for one hour at room temperature in order to extract all the ^{3}H-melatonin into the organic phase. This system is a more efficient medium for radioactivity detection than a detergent-based system and is less sensitive to quenching.

The ß-radioactivity in the tubes was counted for 5 minutes using an automatic ß-scintillation counter (Rackbeta, Pharmacia). The counting efficiency of the samples was 25%. The sample concentrations were calculated using a 'RIAcalc' program.

3.7 Physiological Signs of Sexual Maturity in Gerbils

The areas of the ventral glands: At 10, 16 and 28 weeks of age, the widths and lengths of the ventral glands were measured to the nearest 0.5 mm using callipers. The areas of the glands were estimated by the formula for an ellipse (¼ pi x length x width). To facilitate measurement of the ventral gland, the abdominal hair was dampened with a small amount of water.

The age at vaginal opening: Each female was examined daily for vaginal opening. The age at which vaginal oestrous occurred was recorded.

Body weights of gerbils were recorded (to the nearest gram).

Right and left testes were removed and weighed at time of sacrifice. Combined weights of the testes were used in data analysis.

3.8 Statistical Methods

3.8.1 The Fluoride Contents of Human Tissues

The [F] of pineal (wet), muscle (wet) and bone (ash) and [Ca] of pineal (wet) were expressed as mean ± SD. The amount of hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, in the pineal was determined as follows:- MW of HA = 1004, therefore HA = total calcium x 1004/400, HA = total calcium x 2.5. The [F] of HA was expressed as mean ± SD. The degree of calcification was determined by expressing the weight of pineal HA as a percentage of the original weight of pineal.

Statistical differences between the mean [F] in pineal and muscle (wet weight) were determined using unpaired Student's *t*-test (two tailed). Pearson's correlation coefficients were used to test correlations between: i) pineal [F] and pineal [Ca]; bone ash [F] and pineal [F]; iii) pineal weight and degree of calcification. Levels of significance were set at $p < 0.05$.

3.8.2 Urinary aMT6s Levels as an Index of Pineal MT Synthesis

Data were expressed as pg MT/pineal (mean ± SD). The statistical significance of differences in the pineal MT contents at different times of the 24-h cycle were determined by one way ANOVA. Pearson's correlation coefficient between pineal MT contents at 0400 and corresponding total 24-h excretion of urinary aMT6s was determined. Levels of significance were set at $p < 0.05$.

3.8.3 Urinary Levels of aMT6s in Gerbils

Results were expressed in ng aMT6s/ml urine. The value of aMT6s in ng/ml was multiplied by the volume of urine from which the aliquot had been taken to provide the value for aMT6s excretion during the time period of the collection; either as ng aMT6s/3-h or ng aMT6s/24-h. aMT6s excretion was also expressed as a function of body weight (BW): either as pg aMT6s/g BW/3-h or pg aMT6s/g BW/24-h.

As the urine samples were collected over two consecutive days, each aMT6s value for the purposes of statistical analyses is the mean of two separate 24-h collection values. However, to make the statistical analyses more stringent, in those instances when the total urinary aMT6s were significantly higher in day 1 than day 2, (CV% > 10%), the levels in day 1 were used in the data analysis rather than the mean of two consecutive days.

One-way analysis of variance (ANOVA) was used to determine differences between groups. In the longitudinal study, where the same individuals were monitored at 7, 9, 11½ and 16 weeks, repeated ANOVA followed by multiple comparisons with Bonferroni correction were used. Total 24-hour excretion of urinary aMT6s, treatment, (high-F or low-F), time, and sex were analysed by one-way analysis of variance. Comparisons of aMT6s excretion between sexes and groups were performed using unpaired Student's *t*-test (two tailed). The power of a *t*-test (with a 5% risk of a Type 1 error) to detect the observed mean difference in aMT6s excretion between the groups or

sexes was calculated. The 95% confidence interval for the difference in aMT6s excretion rates between the sexes or groups was calculated. Levels of significance were set at $p < 0.05$.

The data from the combined longitudinal and additional gerbils was analysed by ANOVA for the overall tests of significance followed by Student-Newman-Keuls test for differences among means.

3.8.4 The Circadian Rhythm of Urinary aMT6s in Gerbils Monitored at 11½ and 16 Weeks of Age

The following adjustments were made to the circadian data in order to improve the statistical analysis:

i) If a gerbil did not void during a 3-h interval, the value for aMT6s in the subsequent 3-h interval was averaged over 6-h.

ii) if a gerbil did not void during the last 3-h interval (16th) the amount of aMT6s/3-h excreted was estimated (1-2 ng). iii) if the total 24-h aMT6s was substantially higher in day 1 than the total day 2, i.e., CV% between the levels of aMT6s excreted in day 1 and day 2 were greater than 10%, the circadian data determined during day 1 were repeated for day 2 and the latter data were ignored.

The data were expressed as ng aMT6s/3-h or pg aMT6s/g BW/3-h. The mean ± SD excretion rates of aMT6s by male and female gerbils from the HF and LF groups were computed over

48-h at 11½ weeks (for statistical analyses, the mean aMT6s/3-h values over 24-h were the mean of the mean aMT6s/3-h over 48-h by gerbils); over 24-h at 16 weeks. At 11½ and 16 weeks, n = 11 for HF females, n = 12 for HF males, LF males and LF females. The mean aMT6s circadian profiles were statistically analysed using unpaired Student's *t*-test (two tailed) to compare aMT6s excretion at 3-h time intervals between sexes and groups. Results were considered significant when below the 0.05 level.

3.8.5 The Physiological Signs of Puberty in Gerbils

The difference between the areas of the ventral glands in the groups were statistically analysed using the two-tailed Fisher exact probability test. Comparisons of the body weights of the gerbils at specific ages were performed using two-tailed Student *t*-tests. Difference between the ages at which vaginal opening occurred in the groups was analysed using Chi-square test statistic, applying Yates correction. Statistical differences between the mean combined testes weights in LF and HF males were determined using unpaired Student's *t*-test (two tailed).

3.8.6 Description of the Experimental Groups

Statistical differences between the mean [F] of gerbil bone ash at specified ages in LF and HF males were determined using unpaired Student's *t*-test (two tailed).

CHAPTER 4 - Fluoride Contents of Human Tissues

4.1 Results

Table 4.1 presents the F-content of human pineal gland and corresponding muscle and bone ash. The data for pineal and muscle are reported on a wet weight basis. The mean [F] (± SD) of the pineal, muscle and bone ash were 296 ± 257 mg/kg (14 to 875 mg/kg), 0.5 ± 0.4 mg/kg (0.2 to 1.5 mg/kg) and 2037 ± 1095 mg/kg (838 to 3711 mg/kg) respectively.

Table 4.1 The fluoride contents of human pineal gland and corresponding muscle and bone ash.

No	pineal (wet wt) mg	pineal total [F] µg	pineal (wet wt) mg F/kg	bone (ash) mg F/kg	muscle (wet wt) mg F/kg
1	63	5	83	3607	0.7
2	157	43	274	1256	0.2
3	154	90	585	1430	0.3
4	91	17	190	910	0.4
5	198	41	209	1475	1.5
10	67	24	356	3711	0.2
12	154	135	875	838	0.2
13	62	4	57	2123	0.8
14	155	68	440	1162	0.3
15	56	1	14	2566	0.3
16	75	13	178	3329	0.4

The mean coefficient of variation between the replicates for F-contents of bone samples was 2.5 ± 1.1%, (± SD, n = 6).

Statistical evaluation of the data showed that the mean pineal [F] was significantly higher ($p < 0.001$) than mean muscle [F]. There was no correlation between [F] of bone ash and [F] of pineal (wet).

Table 4.2 presents the [Ca], the estimated [HA], and estimated [F] of HA within the aged human pineal glands. The [F] of HA in the pineal no. 5 was too high and was not included in the subsequent data analysis.

Table 4.2 The calcium concentration and estimations of the amount of hydroxyapatite and the F-concentration in the hydroxy-apatite in aged human pineal glands.

no	Ca^{++} pineal µg	$[Ca^{++}]$ pineal (wet) mg/kg	pineal [HA] mg	total F pineal µg	[F] in PC mg/kg HA
1	1115	17 780	3	5	1750
2	831	5300	2	43	20 490
3	5722	3 7270	14	90	6290
4	2691	29 570	7	17	2490
5	280	1410	1	41	59 330
10	846	12 730	2	23	10 920
12	3740	24 240	9	135	14 450
13	286	4640	1	3	4650
14	1248	8040	3	68	21 780
15	446	7970	1	1	650
16	928	12 410	2	13	5560

N.B. The amount of hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$, in the pineal was determined as follows:- MW of HA = 1004, therefore HA = total calcium x 1004/400, HA = total calcium x 2.5

The mean concentration of calcium within the pineal gland (wet) was 16 000 ± 11 070 mg/kg (± SD) ranging from 4600 to 37 300 mg/kg. The mean percentage of the weight of the pineal (wet)

derived from HA was 4.0 ± 2.8% (± SD) ranging from 1.2 to 9.3%. The mean [F] of the pineal HA was 8900 ± 7700 mg/kg (± SD) ranging from 650 to 21 800. A significant correlation was found between the [F] and [Ca] of the pineal (wet) using Pearson's correlation test: $r = 0.73$: $p < 0.02$. There was no correlation between the degree of calcification and pineal (wet weight): $r = 0.3$, $p < 0.4$. The mean weight of the pineal gland was 112 ± 52 mg (± SD) ranging from 56 to 198 mg.

See Appendix A for results.

4.2 Conclusions

The aged human pineal contained high levels of F although there was considerable inter-individual variation in the amount of F retained (ranging between 14-875 mg/kg). In direct contrast, muscle consistently contained very low levels of F, a typical result for soft tissue. There was a highly significant difference ($p < 0.001$) between the mean [F] of pineal and muscle (wet weights): 296 ± 257 *vs*. 0.5 ± 0.4 mg/kg respectively. If the T/P ratio for F in the pineal had been similar to that reported in brain, i.e., 0.08 (Whitford *et al*, 1979), then the [F] of pineal would be equivalent to, or less than, that of muscle. This was obviously not the case which proves that the human pineal is outside the blood-brain barrier with respect to F.

The human pineal gland contains more F than any other normal soft tissue. The mean [F] in the pineal (wet weight) was more than 300 times higher than in human kidney, which was considered to

have the highest [F] of all normal soft tissues: 0.7 mg/kg (wet weight) (Gettler and Ellerbrook, 1939). The pineal hydroxyapatite contained extremely high levels of F, i.e., the mean [F] of PC was four times higher than the mean [F] in bone ash: 8900 *vs*. 2040 mg/kg respectively. The complete substitution of OH in the apatite crystal lattice by F would convert the mineral to fluorapatite which contains 38 000 mg F/kg. This transformation is never achieved in human calcified tissues. Nevertheless, the degree of substitution of OH in pineal hydroxyapatite by F was sometimes extremely high, e.g., nos. 2 and 14 contained 20 490 and 21 780 mg F/kg respectively. This may be due to the pineal's copious blood flow and capillary density, and the large surface area of the crystallites. The pineal also contains very high levels of zinc, iron, manganese, magnesium and copper (Michotte *et al*, 1977).

The result of the F-concentration of HA in pineal no. 5 was too high (59 330 mg/kg) unless F accumulates within the pineal by associating with constituent(s) other than calcium. I am confident that the recorded F-concentration of pineal no. 5 was satisfactory (209 mg/kg). This recorded value may even have been lower than its true value because, being the first pineal to be analysed, no. 5 had not been divided into two portions and consequently the trapping solution may not have been strong enough to trap all the diffused F. The error in the [F] of HA probably occurred prior to the determination of its calcium content: maybe during the storage of the acid digests.

The pineal is an intriguing organ because it can be classified either as a soft or as a mineralizing tissue. The percentage of HA in the

aged pineal varied eight-fold: 1.2 to 9.3%. Two of the ten aged pineals (nos. 13 and 15) in the current study contained very little calcification which is consistent with previous studies (Cooper, 1932; Arieti, 1954; Hasegawa *et al*, 1987; Galliani *et al*, 1990). This suggests that the presence of PC may not be directly associated with atrophy or age of the subject.

The mean [F] of bone ash from subjects (mean age of 82 years) was 2037 mg/kg. This result is in agreement with a previous study (Ebie *et al*, 1992) which reported that the mean [F] of human bone ash (mean age of 72 years) was 1834 mg/kg. There was a wide variation in the [F] of bone ash from subjects of similar age. This may been partly caused by: i) different dietary habits of the subjects; ii) different types of bone were analysed. With hindsight, I should have removed the same type of bone from each cadaver for F-analysis because different types of bone contain varying amounts of F (Jenkins, 1990). There were not enough subjects to be able to correlate bone [F] with age.

The mean (± SD) weight of the human pineal (wet) in this study was 112 ± 52 mg (range of 56-198 mg). This result is in agreement with Hasewaga and co-workers, (1987), who reported that the mean weight of formalin-fixed, aged human pineals was 95 ± 49 mg (18-346 mg). There was no correlation between pineal weight and the extent of calcification ($r = 0.3$, $p < 0.4$) which agrees with Tapp and Huxley, (1971).

No correlation was found between the [F] in bone ash and pineal gland (wet). Therefore, the amount of F deposited in the pineal

gland is not an index of previous F-intake, unlike bone ash. This suggests that F-intake is not directly associated with the F-retention by the pineal. A significant correlation was found between pineal [Ca] and pineal [F] (wet weight): using Pearson's correlation test, $r = 0.71$, $p < 0.02$. There is a direct relationship between pineal calcium and pineal F, although which is deposited first remains unanswered. There are a number of calcium-dependent steps in the biosynthesis and release of MT. On a molecular level, calcium has inhibitory or activating properties on various enzymes which balance catalytic activity. The binding of calcium by F could affect the biosynthesis of MT.

An increase in the F-content of pineal hydroxyapatite will lead to an increased stability of the mineral. This may be a disadvantage if PC are labile, dynamic structures as suggested by Champney and co-workers, (1985). Welsh and Beitz (1981) suggested that PC functions as a storage site for some pineal product (peptides) which are used when their synthesis no longer keeps up with demand. If this is so, a less soluble apatite may hinder this process.

A further study is recommended to determine the F-levels in children's pineals. It is unlikely that they will contain such high levels of F as the pineals from elderly subjects because the PC in children is usually more subtle than in adults. Nevertheless, high levels of trace elements have been demonstrated in the so-called 'uncalcified' pineals (Michotte *et al*, 1977; Humbert and Pévet, 1991). A recent study showed that the mean peak [F] of developing enamel organ from a 9-day-old rat pup was less than 0.2 mg F/kg following an oral dose of 0.5 μg F/g BW (Bawden *et al*, 1992). If

equivalent levels of F are present in young children's pineals they may interfere with a much broader spectrum of cellular processes than those in the enamel organ.

The physiological mechanism, which prevents the passage of F from plasma into human milk, was presumably evolved to exclude any effects of F on the infant. This begs the question as to which tissue needs protecting. The mechanism is possibly directed towards the protection of a calcifying tissue because F is only attracted to and retained in areas which are undergoing calcification. The bones do not require protection because F is deposited in them in utero. It may be that the mechanism serves to protect the subtle calcification in the pineal from the effects of F.

Previous investigators have obviously neglected to dissect the pineal from the brain for the determination of its F-content. This may seem surprising because it is a simple procedure and the pineal calcification can reach macroscopic proportions (looks like sand). In conclusion, the results from this study show for the first time that human pineal contains the highest levels of F out of all soft tissues. Therefore, the pineal may be a hitherto unrealized target for chronic F-toxicity.

CHAPTER 5 - Urinary Levels of aMT6s as an Index of Melatonin Synthesis in the Gerbil Pineal

5.1 Results

The intra-assay coefficients of variation for the RIA for melatonin were 9.6%, 4.9% and 6.3% at 37, 117 and 319 pg MT/0.5 ml respectively. The inter-assay coefficients of variation were 13.9%, 3.3% and 9.6% at 27, 88 and 280 pg MT/0.5 ml respectively. See table B.1 in Appendix B. The limit of sensitivity of the assay was 2.2 pg MT/ml.

Table 5.1 Melatonin contents in gerbil pineals (pg/pineal) at 16 weeks at 6-hourly intervals throughout 24-hours.

Time	mean ± SD	SEM	n
10:00 am	96 ± 40	10	15
4:00 pm	109 ± 43	11	14
10:00 pm	136 ± 84	24	12
4:00 am	683 ± 421	99	18

Table 5.1 presents the mean (± SD, ± SEM) melatonin content in gerbil pineal glands aged 16 weeks (pg MT/gland) at 6-h intervals throughout the 24-h cycle. The gerbil pineal contained the highest MT levels (683 ± 421 pg/pineal) at 0400, nine hours after the onset of darkness. See figure 5.2. The mean 0400 pineal MT content was significantly higher ($p < 0.001$) than the mean daytime pineal MT content (103 ± 41 pg MT/gland). There was no significant

difference between the pineal MT contents at daytime 1000, daytime 1600 and night-time 2200: df = 2, 38, F = 1.69, $p < 0.2$.

See tables B.2 and B.3 for laboratory results.

Validation of the Use of Subjects

The mean pineal MT contents at 1000, 1600, 2200 and 0400 were computed from random samples of 16-week-old male and female gerbils from three groups: HF, LF and LFNM. Therefore, before the pineal MT contents could be compared with urinary aMT6s excretion rates, it was necessary to determine whether there was a significant difference between the excretion rates of urinary aMT6s by the groups and the sexes.

The intra-assay coefficients of variation for the RIA for urinary aMT6s were 4.3%, 8.2% and 5.7% at 3.5, 12.3 and 24.3 ng aMT6s/ml respectively. See table C.1. The inter-assay coefficients of variation for the RIAs for urinary aMT6s excreted by the LF, HF and LFNM groups at 16 weeks were 16.3%, 6.0%, and 9.3% at 3.4, 14.2 and 24.0 ng aMT6s/ml. See table B.4.

Figure 5.1 clearly illustrates the similarity between the mean circadian profiles of urinary aMT6s (pg/g BW/3-h) in 12 male and 12 female gerbils from the three groups. A high-F intake had no effect on the duration or the amplitude of the nocturnal cycle of urinary aMT6s excretion. In addition, there was no significant difference between the total urinary aMT6s (pg/g BW/24-h) excreted by male and female gerbils from the three groups: F =

0.58, df = 5, 65, $p < 0.7$. This justified the pooling of data for pineal MT contents and for circadian profiles of urinary aMT6s in male and female gerbils from the three groups.

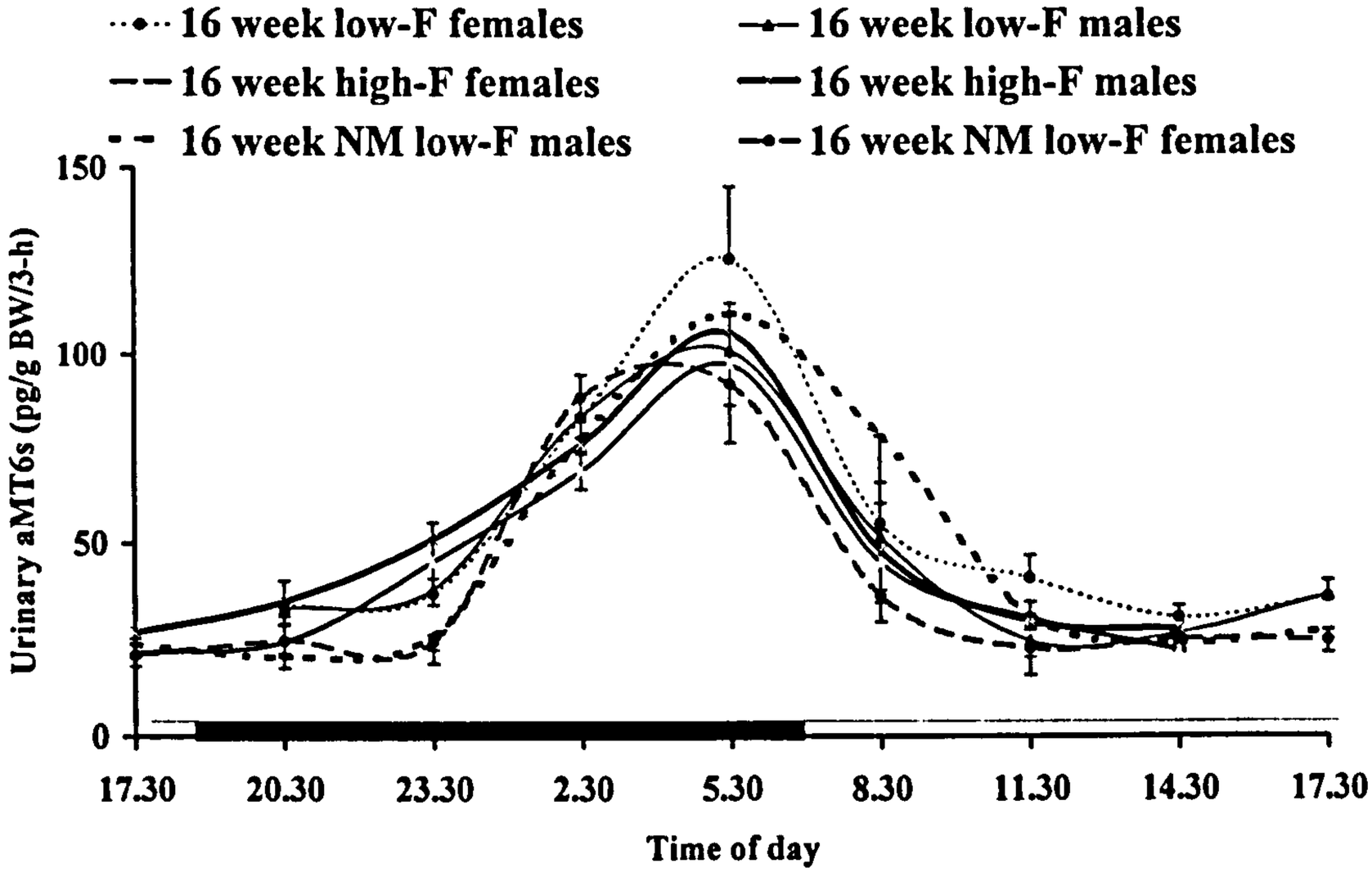

Fig. 5.1 Mean circadian profiles of urinary aMT6s by male and female gerbils from the HF, LF and LF non-monitored groups at 16 weeks, n = 12. The black bar represents the period of darkness; labels on the x-axis represent the mid-points of the 3-h urine sampling intervals; vertical lines above (or below) the data points represent SEM.

Figure 5.2 presents the mean pineal MT content (n = 12-18, ± SEM) at 6-h intervals and the mean (± SEM) 3-h excretion rate of urinary aMT6s (pg/g BW/3-h) by 16-week-old gerbils (n = 71) over a 24-h period. There was a clear diurnal rhythm in urinary aMT6s excretion with low values during the daytime and high values during the night. The maximum rate of urinary aMT6s excretion was 117 ± 65 pg aMT6s/g BW/3-h (mean ± SD) and this occurred between 0400 to 0700. The mean amplitude of the aMT6s signal

(peak value minus mean daytime value) was four-fold. The rate of urinary aMT6s excretion was 28 ± 13 pg aMT6s/3-h (± SD) during the daytime and early dark period. The first pool of urine after lights-on (0700-1000) had elevated levels of urinary aMT6s.

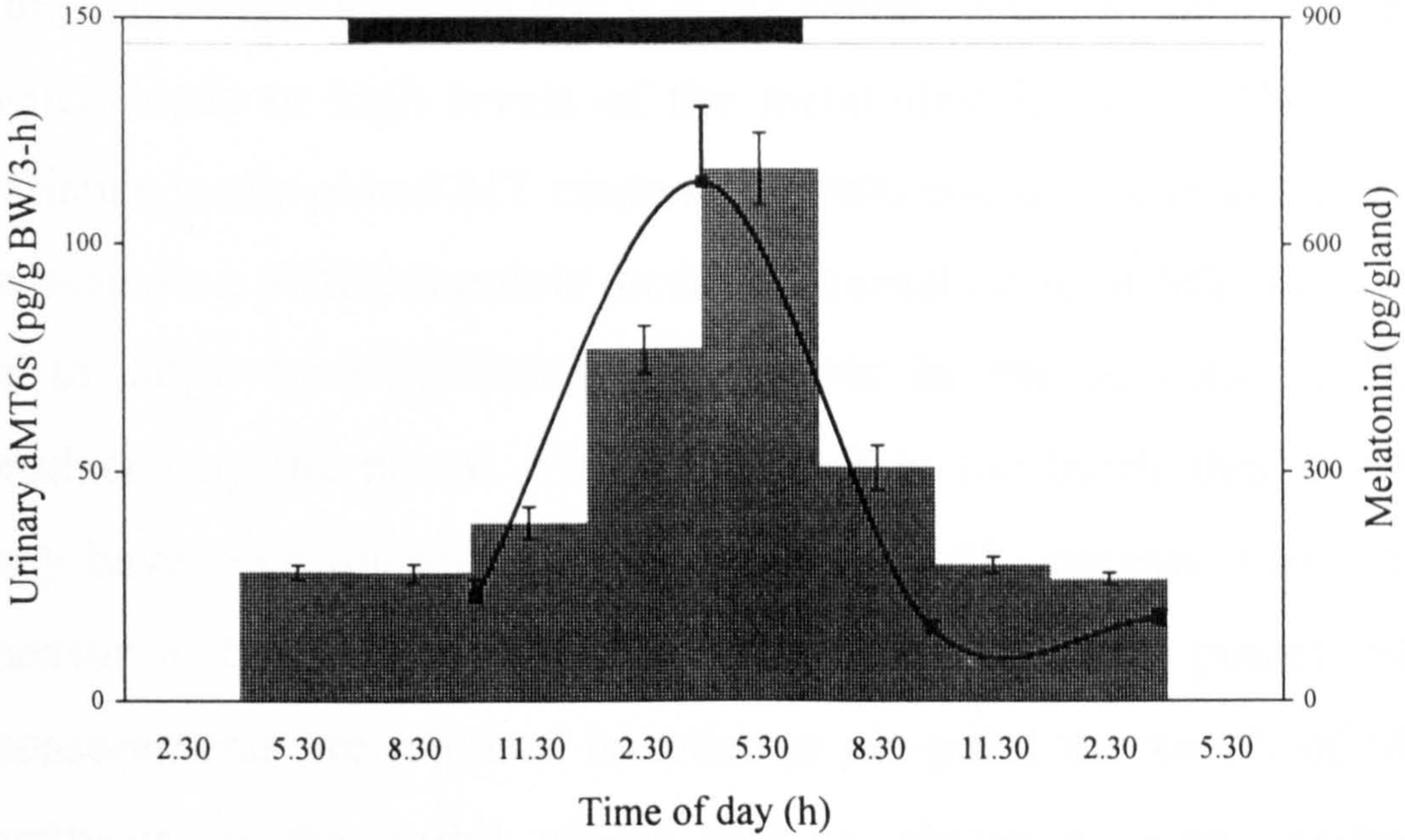

Fig. 5.2 Mean (± SEM) melatonin content in gerbil pineal glands aged 16 weeks are plotted *vs*. clock time with units of measurement indicated to the right. On the same time axis, the mean (± SEM) urinary aMT6s excretion rate (pg/g BW/3-h) is given as a histogram. Horizontal bar indicates period of darkness.

The peak (0400) nocturnal values for gerbil pineal MT contents at 16 weeks were correlated to the corresponding excretion rates of urinary aMT6s (pg aMT6s/g BW/24-h) expressed as a function of body weight rather than the absolute levels because the males were significantly heavier than the females. Pearson's correlation coefficient between the pineal MT content at 0400, (pg MT/pineal), and the corresponding total urinary aMT6s (pg/g BW/24-h) was 0.73 ($p < 0.05$). See table B.5 in Appendix B.

5.2 Conclusions

At 16 weeks, the melatonin profile in the gerbil pineal presented essentially the same day/night pattern as the profile of urinary aMT6s. The increase in the levels of pineal MT and its metabolite during the night suggests that it is the secretion of MT by the pineal which leads to high levels of the metabolite in urine. The large variance in the pineal MT contents at 0400 could be due to the data representing different points on the nocturnal cycle of MT secretion or to large inter-individual differences in the amount of MT produced by the pineal. The exact peak in the biorhythm of MT may have been missed because the pineal MT contents were only measured twice during the night. More frequent pineal MT measurements are required in order to pin-point the zenith of MT synthesis in the gerbil pineal and so obtain a more accurate assessment of the pineal MT profile.

The shape of the MT profile, its duration and the fact that gerbil pineal secretes the highest levels of MT late in the night are consistent with the gerbil pineal being classified as having a type A pattern of MT secretion (Reiter, 1987). The six-fold amplitude (night-time *vs*. daytime) of the pineal MT content in 16-week-old gerbils in this study is not consistent with the study (Reiter *et al*, 1980) which reported a two-fold amplitude in pineal MT content in 8-week-old male gerbils and no nocturnal increase in pineal MT output by the aged gerbil. Rudeen and co-workers (1975) reported a three-fold amplitude in NAT activity in the gerbil pineal with the highest enzyme activity at 0400, eight hours after lights-out (LD:

14 10). However, neither the age nor the sex of the gerbils was reported.

The urinary aMT6s profile was delayed in time compared to the pineal MT profile. For example, aMT6s levels were still high during the first 3-h interval after lights-on (0700-1000) even though it has been well established that pineal MT synthesis is quickly suppressed following light exposure. The presence of high values of urinary aMT6s excreted during 0700-1000 was presumably caused by the time required for MT to be metabolized to aMT6s in the liver and the time that urinary aMT6s was retained in the bladder. In addition, in order to compensate for the few 3-h intervals when some gerbils did not void, the aMT6s values excreted in the subsequent 3-h interval were averaged out over six hours. Therefore, the recorded values for urinary aMT6s excreted during the early hours of the dark period were probably higher than the true values. Nevertheless, all gerbils had a pattern of urinary aMT6s excretion which was tightly coupled to the light/dark cycle.

At 16 weeks of age, a high-F intake had no effect on the circadian profiles of urinary aMT6s excretion by gerbils; nor was there a sex difference. The 16-week-old gerbils excreted 28.3 ± 1.3 ng aMT6s/24-h (mean ± SEM) under LD: 12 12. This is similar to the total amount of urinary aMT6s excreted by the Djungarian hamster (12 males, 12 females, aged 3-7 months) under LD: 8 16: 29.8 ± 4.3 ng/24-h (Stieglitz *et al*, 1995).

In the few species that have been studied so far, MT is metabolized predominantly, (although not exclusively), to urinary aMT6s.

Depending upon the species, however, the metabolic pathway of MT may vary. Further studies are required to discover the percentage of pineal MT which is metabolized to urinary aMT6s in the gerbil. If the pineal MT contents were determined more frequently throughout the 24-h cycle, the daily pineal output of MT could be calculated using the method described by Rollag *et al*, (1980). The daily pineal output of MT could then be compared with total urinary aMT6s levels.

There was a significant correlation ($r = 0.73$, $p < 0.05$) between pineal MT content at 0400 and the corresponding total urinary aMT6s; that the correlation was not higher may be due to experimental limitations. This finding agrees with previous studies: (Markey *et al*, 1985) who found a significant correlation between total urinary 6-hydroxymelatonin and night-time plasma MT concentrations in humans; (Arendt *et al*, 1985) who reported a significant correlation between the area under the curve (AUC) for the 24-h profile of plasma MT and total urinary aMT6s in humans; and Stieglitz *et al*, (1995) who showed that total urinary aMT6s was significantly correlated with nocturnal pineal MT content in the Djungarian hamster.

In conclusion, the diurnal rhythm in the MT content in the gerbil pineal is mirrored in the rate of urinary aMT6s excretion. The levels of urinary aMT6s, as measured by RIA, reflect the general temporal pattern of pineal MT synthesis and represent a quantitative index of pineal activity in the gerbil.

CHAPTER 6 - Effects of Fluoride on Total Urinary aMT6s Excretion by Gerbils

6.1 Results of the Longitudinal Study of Total Urinary aMT6s Excreted by Gerbils at 7, 9, 11½ and 16 Weeks of Age.

The intra-assay coefficients of variation (CV%) were 4.3%, 8.2% and 5.7% at 3.5, 12.3 and 24.3 ng aMT6s/ml respectively. See table C.1 in Appendix C. The inter-assay CV% were 4.7%, 5.2% and 9.2% at 3.7, 13.7 and 23.0 ng aMT6s/ml respectively. See table C.2. The limit of sensitivity of the assay was 0.2 pg aMT6s/ml. See table C.5.

Tables C.6, C.7 and C.8 list the CV% between the levels of urinary aMT6s excreted in day 1 and day 2. The mean CV% was 14.3 ± 14.3%, median was 10.9%, 25th percentile was 5.0%, 75th percentile was 20.0%. Tables C.6, C.7 and C.8 show that out of the 78 instances where the CV% was greater than 10%, seven instances occurred which were due to substantially higher levels of aMT6s in day 1 than day 2. In these seven instances, the aMT6s levels in day 1 were used for data analyses; whereas for the rest, each aMT6s value is the mean of the levels of urinary aMT6s in day 1 and day 2. At 16 weeks, the mean values were the means of one 24-h total aMT6s excreted by 12 males or 12 females from both groups. One HF female (no. 2) died at 14 weeks. Unfortunately, I did not

determine the cause of its death. Its data at 11½ weeks were not included in the statistical analyses.

Table 6.1 presents the mean (± SD) urinary aMT6s levels excreted in 24-h by 12 females and 12 males from the HF and LF groups at 7, 9, 11½ and 16 weeks of age, i.e., spanning the time of sexual maturation in gerbils. At all ages, the LF gerbils excreted more total urinary aMT6s than the HF group; irrespective of whether the data were expressed as ng/24-h or pg/g BW/24-h.

Figure 6.1 presents the mean total aMT6s excreted by gerbils from the HF and LF groups as bar charts with vertical bars above the columns representing the SEM and asterisks representing the significance levels. The bar charts clearly show that the HF group excreted significantly lower levels of aMT6s than the LF group up until the time of sexual maturation in this species.

At 7 weeks, the LF males excreted almost twice as much urinary aMT6s/24-h as the HF males: 30.7 ± 7.9 *vs.* 16.4 ± 4.2 ng/24-h (± SD): $p < 1.5E-05$. The power of a t-test (with a 5% risk of a Type 1 error) to detect the observed mean difference in aMT6s excretion was 100%. The 95% confidence interval for the difference in aMT6s excretion rates between the HF and LF males was 8.9 to 19.7 ng/24-h.

At 7 weeks, the LF females also excreted significantly higher levels of aMT6s/24-h than the HF females: 26.8 ± 6.8 *vs.* 18.1 ± 5.5 ng/24-h (± SD): $p < 0.002$. The power was 0.97; the 95% confidence interval was 3.5 to 13.9 ng/24-h.

Table 6.1 Summary of mean urinary aMT6s levels excreted in 24 hours by gerbils monitored at 7, 9, 11½ and 16 weeks of age. Data expressed as mean ± SD, n = 12. Data from a high-F female which died at 14 weeks of age were not included at 11½ weeks

	AGE weeks	LOW-FLUORIDE FEMALES	HIGH-FLUORIDE FEMALES	LOW-FLUORIDE MALES	HIGH-FLUORIDE MALES
		MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD
mean aMT6s ng/24-h	7	26.8 ± 6.8	18.1 ± 5.5	30.7 ± 7.9	16.4 ± 4.2
	9	25.6 ± 6.4	20.2 ± 6.2	27.9 ± 7.7	19.6 ± 4.7
	11½	27.7 ± 7.1	26.1 ± 9.5	33.0 ± 9.8	21.9 ± 5.7
	16	29.8 ± 8.2	24.2 ± 11.6	34.1 ± 14.5	31.6 ± 10.9
mean aMT6s pg/g BW/24-h	7	602 ± 168	359 ± 109	569 ± 148	308 ± 76
	9	476 ± 109	365 ± 106	425 ± 113	320 ± 75
	11½	468 ± 102	407 ± 134	449 ± 111	299 ± 74
	16	443 ± 126	346 ± 147	397 ± 148	399 ± 112
body weight g	7	45 ± 3.0	51 ± 5.6	54 ± 3.0	54 ± 5.8
	9	54 ± 3.4	55 ± 4.0	66 ± 4.9	61 ± 3.5
	11½	59 ± 5.9	63 ± 6.3	73 ± 6.9	73 ± 3.8
	16	67 ± 5.5	68 ± 9.9	85 ± 9.5	78 ± 6.1

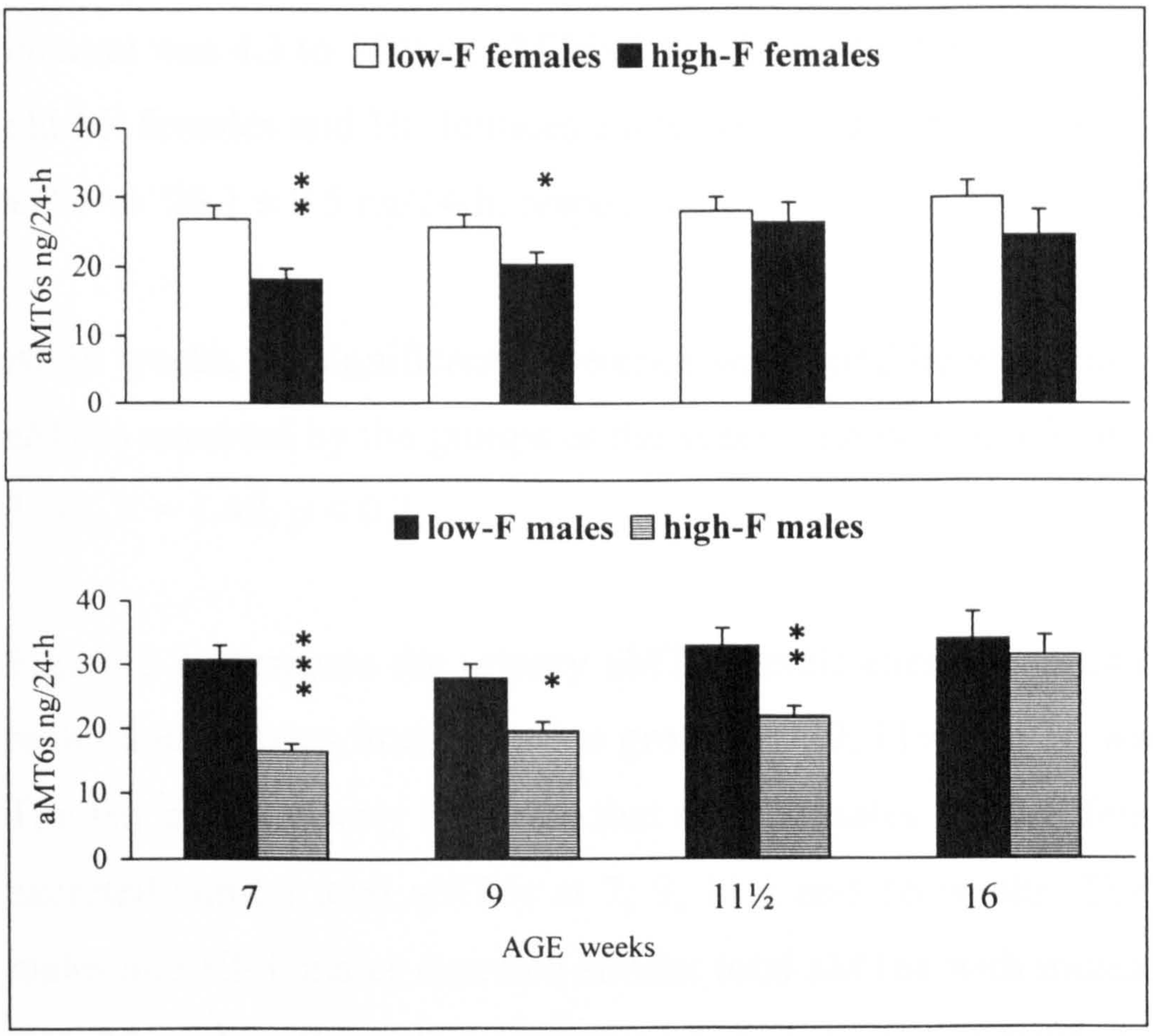

Fig. 6.1 A comparison of the absolute levels of urinary aMT6s excreted by gerbils from the HF and LF groups monitored at 7, 9, 11½ and 16 weeks. LD: 12 12. Data expressed as mean ± SEM, n = 12; *p < 0.05, **p < 0.005, ***p < 0.00001

At 9 weeks, the LF males continued to excrete significantly higher levels of aMT6s/24-h than the HF males: 27.9 ± 7.7 *vs.* 19.6 ± 4.7 ng/24-h (± SD) respectively: $p < 0.004$. The power was 0.9; the 95% confidence interval was 2.9 to 13.7 ng aMT6s/24-h. At 9 weeks, the LF females excreted 25.6 ± 6.3 ng aMT6s/24-h *vs.* 20.2 ± 6.2 ng aMT6s/24-h by the HF females: $p < 0.05$. The power was 0.6; the 95% confidence interval was 0.1-10.7 ng aMT6s/24-h.

At 11½ weeks, the LF males excreted significantly more total aMT6s than the HF males: 33.0 ± 9.8 *vs.* 21.9 ± 5.7 ng/24-h (± SD) respectively: $p < 0.003$. The power was 0.95; the 95% confidence

interval was 4.3 to 17.9 ng aMT6s/24-h. In contrast, the 11½-week-old LF females and HF females excreted similar total aMT6s: 27.7 ± 7.1 *vs*. 26.1 ± 9.5 ng/24-h, respectively.

At 16 weeks, no significant difference was found between the total aMT6s excreted by the groups or the sexes: one-way ANOVA, df = 3, 43, $F = 1.49$, $p < 0.2$.

Figure 6.2 compares the urinary aMT6s levels excreted in 24-h by males and females from the same group at 7, 9, 11½ and 16 weeks. The bar charts clearly illustrate that the LF males and LF females excreted similar total aMT6s at 7, 9, 11½ and 16 weeks. The HF males and HF females excreted similar total aMT6s with increasing age although at 7 and 9 weeks, the levels were significantly lower than the LF group). However, the HF group exhibited different patterns of aMT6s excretion than the LF group: the HF females and HF males had a higher rate of aMT6s excretion after 9 and 11½ weeks respectively.

Using repeated-measures ANOVA:

a) LF females: $F = 2.33$, $v_n = 3$, $v_d = 33$, critical p-value of 2.89.

b) LF males: $F = 3.22$, $v_n = 3$, $v_d = 33$, $p < 0.05$, critical p-value of 2.89. Multiple comparisons with paired *t*-tests, using the residual mean square and the Bonferroni correction, showed that, at 16 weeks, the LF males excreted significantly more aMT6s than at 9 weeks.

c) HF females: $F = 4.94$, $v_n = 3$, $v_d = 30$, $p < 0.01$, critical p-value of 4.51. Multiple comparisons with paired *t*-tests, using the residual mean square and the Bonferroni correction, showed that, at 7

weeks, the HF females excreted significantly less aMT6s/24-h than at 11½ and 16 weeks.

d) HF males: F = 22.82, $v_n = 3$, $v_d = 33$, $p < 0.01$, critical p-value of 4.44. Multiple comparisons with paired *t*-tests, using the residual mean square and the Bonferroni correction, showed that, at 16 weeks, the HF males excreted significantly more aMT6s than at 7, 9 and 11½ weeks. At 16 weeks, the HF males excreted almost twice as much urinary aMT6s/24-h than at 7 weeks: 31.6 ± 10.9 *vs.* 16.4 ± 4.2 ng/24-h, respectively: $p < 0.0002$.

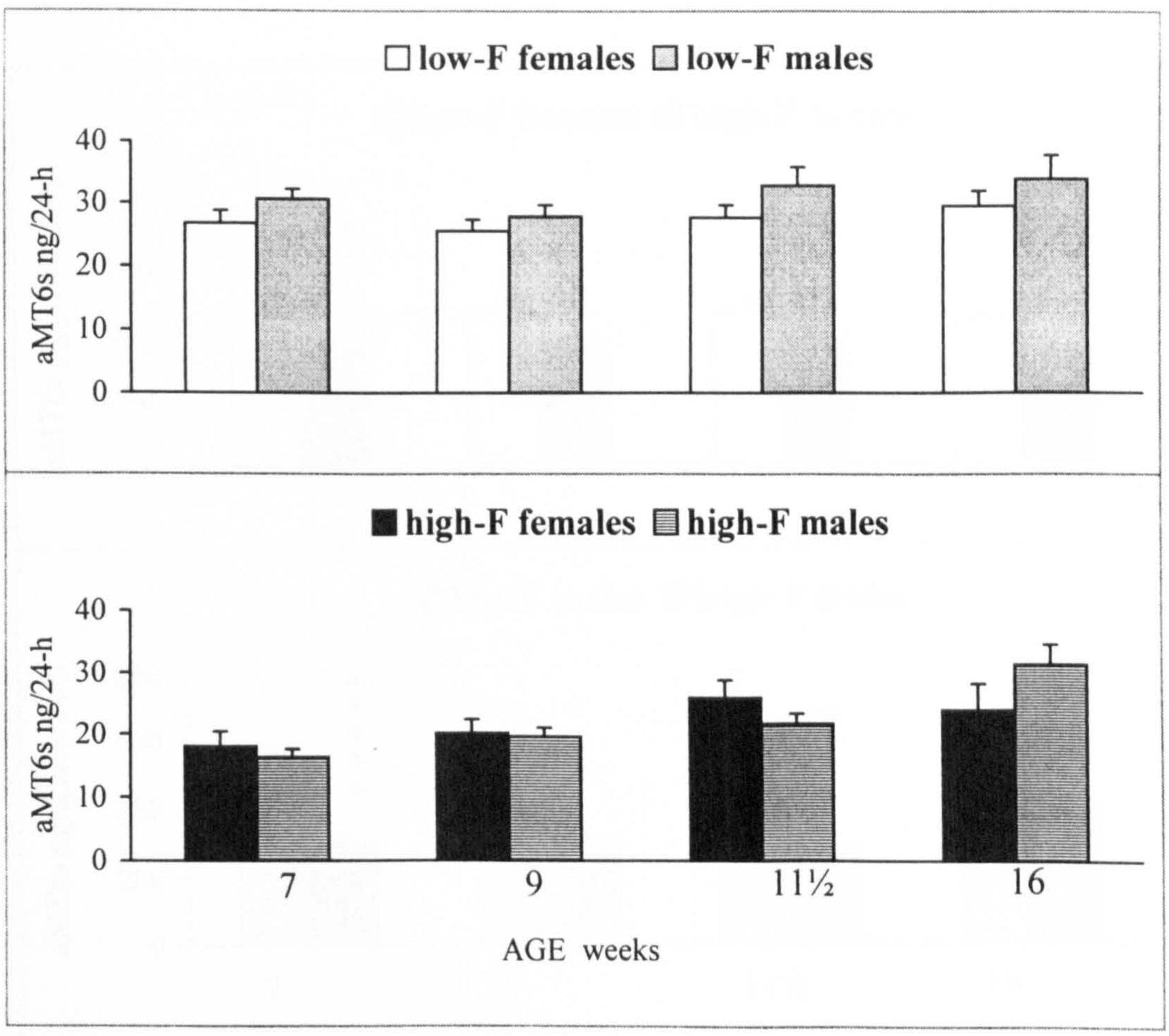

Fig. 6.2 A comparison of the mean absolute levels of urinary aMT6s excreted by male and female gerbils from the same group monitored at 7, 9, 11½ and 16 weeks. LD: 12 12. Data expressed as mean ± SEM, n = 12.

Figure 6.3 illustrates the significant differences between the mean total aMT6s excreted by the HF and LF groups when their body

weights were taken into consideration. At 7 weeks, the LF males excreted almost twice as much urinary aMT6s/24-h as the HF males: 569 ± 148 *vs.* 308 ± 76 pg/g BW/24-h (± SD) respectively: $p < 0.00002$. The power was 100%; the 95% confidence interval was 161 to 361 pg aMT6s/g BW/24-h. At 7 weeks, the LF females also excreted significantly more total aMT6s than the HF females: 602 ± 168 *vs.* 359 ± 109 pg/g BW/24-h, respectively: $p < 0.0004$. The power was 100%; the 95% confidence interval was 123 to 363 pg aMT6s/g BW/24-h.

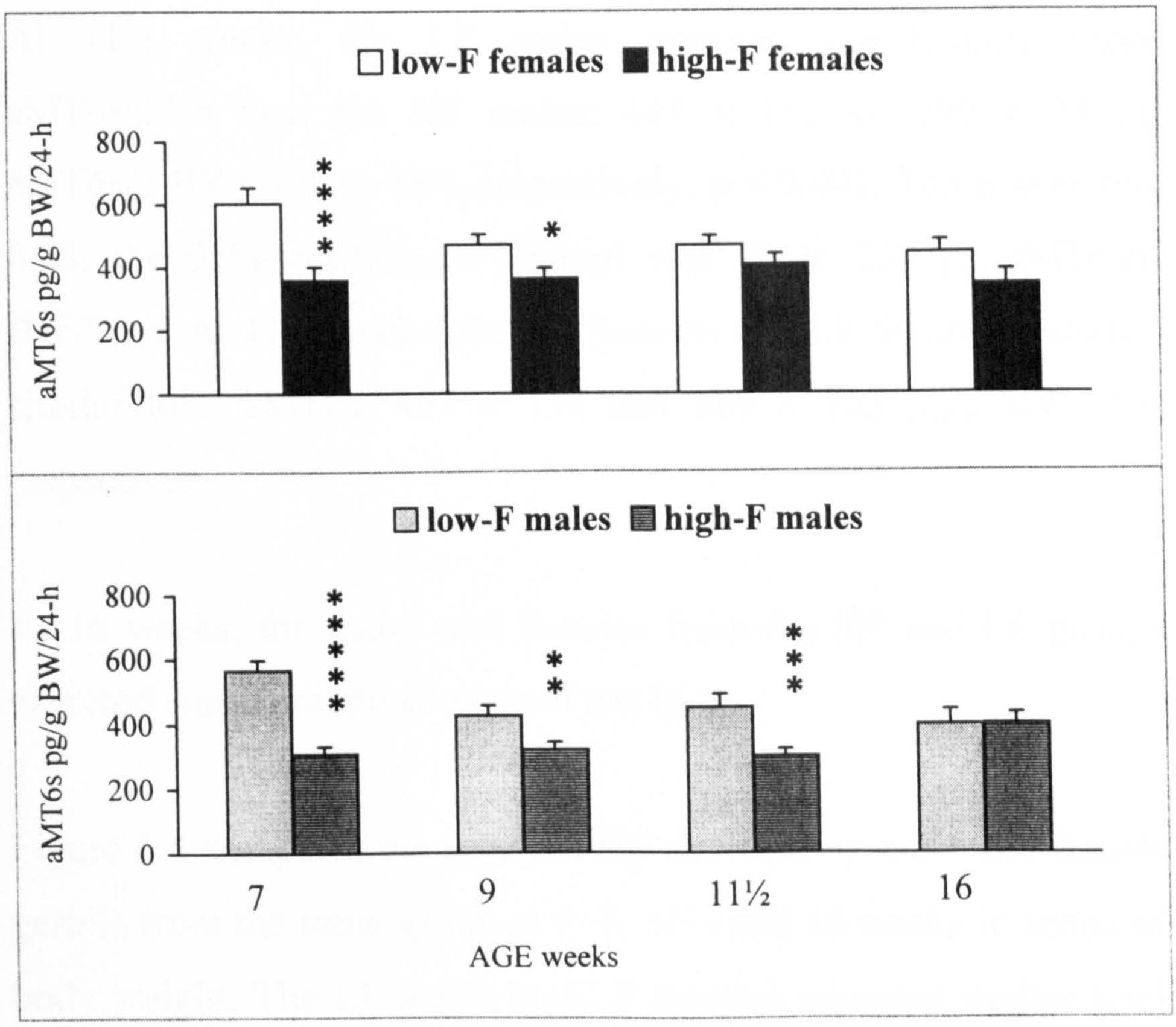

Fig. 6.3 A comparison of the relative levels of aMT6s excreted by gerbils from the HF and LF groups at 7, 9, 11½ and 16 weeks; LD: 12 12. Data expressed as mean ± SEM, n = 12; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0005$, *****$p < 0.00005$.

At 9 weeks, the LF group continued to excrete significantly more urinary aMT6s than the HF group. At 9 weeks, the LF males excreted 425 ± 113 pg/g BW/24-h (± SD) *vs.* 320 ± 75 pg/g BW/24-h (± SD) by the HF males: $p < 0.01$. The power was 0.7; the 95% confidence interval was 24 to 186 pg aMT6s/g BW/24-h. At 9 weeks, the LF females excreted 476 ± 109 pg/g BW/24-h (± SD) *vs.* 365 ± 106 pg/g BW/24-h (± SD) by the HF females: $p < 0.02$. The power was 0.63; the 95% confidence interval was 20 to 202 pg aMT6s/g BW/24-h.

At 11½ weeks, the LF males excreted significantly more aMT6s/24-h than the HF males: 449 ± 111 *vs.* 299 ± 74 pg aMT6s/g BW/24-h (± SD), respectively: $p < 0.001$. The power was 0.98; the 95% confidence interval was 70 to 230 pg aMT6s/g BW/24-h. At 11½ weeks, the HF females and LF females excreted similar total aMT6s: 407 ± 134 and 469 ± 102 pg/g BW/24-h respectively.

At 16 weeks, the males and females from the HF and LF groups excreted similar relative levels of aMT6s.

Figure 6.4 compares the total aMT6s excreted by male and female gerbils from the same group at 7, 9, 11½ and 16 weeks in terms of body weight. The LF males and LF females excreted similar total aMT6s as they passed through puberty to young adulthood. In addition, their patterns of aMT6s excretion were similar, i.e., a decreasing rate of aMT6s excretion with increasing age. The bar charts show that the pattern of aMT6s excretion by the HF group

was significantly different from the LF group throughout puberty. See tables C.12 and C.13.

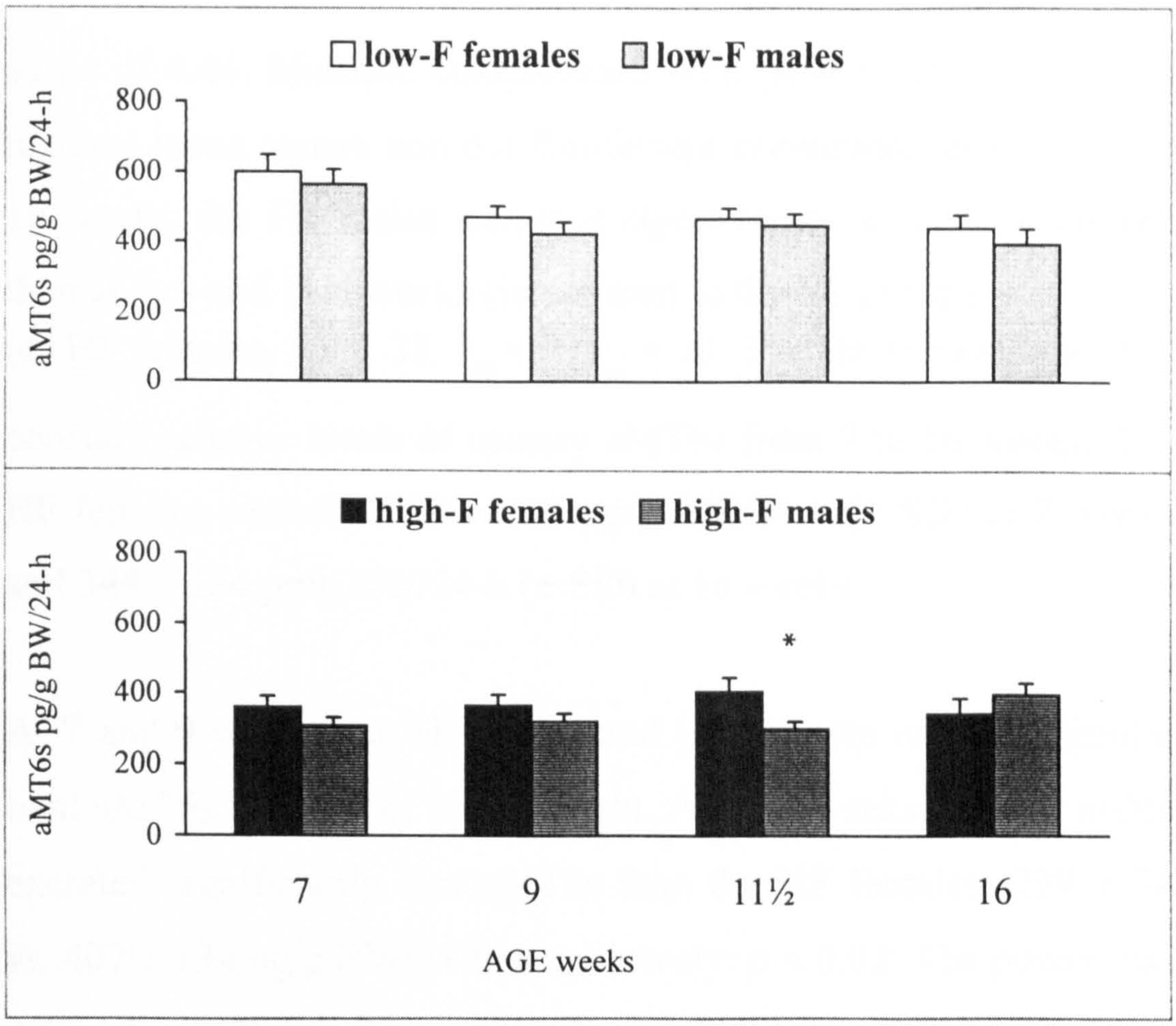

Fig. 6.4 A comparison of the relative levels of urinary aMT6s excreted by male and female gerbils from the same group monitored at 7, 9, 11½ and 16 weeks; LD: 12 12. Data expressed as mean ± SEM; *$p < 0.02$.

Using repeated-measures ANOVA, the relative total aMT6s excreted by the groups at 7, 9, 11½ and 16 weeks were significantly different.

(a) LF females: $F = 7.76$, $v_n = 3$, $v_d = 33$, $p < 0.01$ with critical value of 4.44;

(b) LF males: $F = 15.79$, $v_n = 3$, $v_d = 33$, $p < 0.01$ with critical p-value of 4.44. Multiple comparisons with paired *t*-tests, using the residual mean square and the Bonferroni correction, shows that, at

7 weeks, the LF males and LF females excreted significantly more total aMT6s than at 9, 11½ and 16 weeks.
(c) HF males, F = 7.87, $v_n = 3$, $v_d = 33$, $p < 0.01$ with critical p-value of 4.44. Multiple comparisons with paired *t*-tests, using the residual mean square and the Bonferroni correction, shows that, at 16 weeks, the HF males excreted significantly more total aMT6s than at 7, 9 and 11½ weeks (in contrast to the LF group).
(d) HF females, F = 1.38, $v_n = 3$, $v_d = 30$. The HF females excreted constant relative levels of urinary aMT6s from 7 to 16 weeks. The HF females excreted 359 ± 109 pg/g BW/24-h (± SD) at 7 weeks and 344 ± 154 pg/g BW/24-h (± SD) at 16 weeks.

At 7 and 9 weeks, the HF males and HF females excreted similar total aMT6s in terms of body weight. At 11½ weeks, the HF males excreted significantly less aMT6s than the HF females: 299 ± 74 *vs.* 407 ± 134 pg/g BW/24-h, respectively: $p < 0.02$. The power was 0.63; the 95% confidence interval was 16 to 200 pg aMT6s/g BW/24-h.

At 16 weeks, the HF males and HF females excreted similar relative levels of aMT6s: 399 ± 112 *vs.* 344 ± 154 pg/g BW/24-h respectively.

6.2 Results of Total aMT6s excreted by Gerbils aged 7 to 28 Weeks including Data from Additional Gerbils

The inter-assay CV% for all the RIAs used in this section were 5.3%, 5.3% and 8.8% at 3.7, 13.7 and 23.1 ng aMT6s/ml respectively. See table C.3.

Tables C.9, C.10 and C.11 list the CV% between total aMT6s excreted by the additional gerbils in day 1 and day 2. The data used for subsequent analyses included: (i) total aMT6s in day 1, if day 1 total aMT6s was substantially higher than in day 2, i.e., CV% > 10%; (ii) the mean of two total aMT6s.

The mean CV% between total aMT6s excreted in day 1 and day 2 for all gerbils (including the monitored groups but excluding those whose total aMT6s from day 1 were used) was 12.8 ± 12.1%; median was 9.5%, 25th percentile was 3.9%, 75th percentile was 17.9%.

Table 6.2 Comparison of the data on total aMT6s excreted by gerbils from the longitudinal study and from additional gerbils on a 24-h basis.

aMT6s ng/24-h

	11½ LF females	11½ HF females	11½ HF males	9 HF males	9 LF males
longitudinal (data from 6.1)	26.8 ± 6.8 [12]	26.1 ± 9.5 [11]	21.0 ± 5.7 [12]	19.6 ± 4.7 [12]	27.9 ± 7.7 [12]
additional gerbils	25.7 ± 6.5 [18]	23.0 ± 5.5 [13]	21.5 ± 7.9 [9]	23.8 ± 4.9 [8]	15.3 ± 2.0 [7]

aMT6s pg/g BW/24-h

	11½ LF females	11½ HF females	11½ HF males	9 HF males	9 LF males
longitudinal (data from 6.1)	468 ± 102 [12]	407 ± 134 [11]	299 ± 74 [12]	320 ± 75 [12]	425 ± 113 [12]
additional gerbils	438 ± 128 [18]	375 ± 97 [13]	325 ± 103 [9]	389 ± 86 [8]	250 ± 31 [7]

N.B. Number of animals per group are shown in parentheses.

Table 6.2 presents the two sets of data on total aMT6s taken from: i) the longitudinal study; ii) the additional gerbils (whose urines were collected on a 24-h basis). The two sets of data were similar. Therefore, they were pooled for statistical analyses.

N.B. An additional group of 9-week-old LF males (n = 7) excreted 15.3 ± 2.0 ng aMT6s/24-h. (Table C.31). This is a significantly lower rate of excretion ($p < 0.001$) than the 9-week-old LF males from Section 6.1 which excreted 27.9 ± 7.7 ng/24-h (± SD, n = 12). I concluded that the additional 9-week-old LF males could not be considered as coming from the same population as those in Section 6.1. Their data were not included in the statistical analyses. (See Section 7.2).

Table 6.3 presents the mean (± SD) total aMT6s excreted by all gerbils up until 28 weeks. It includes data from:

(i) Section 6.1, i.e., at 7 weeks, males and females from the HF and LF groups; at 9 weeks, LF males, LF females, and HF females; at 11½ weeks, LF males;

(ii) additional gerbils on a 24-h basis at 9, 11½ and 16 weeks (pooled with data from Section 6.1);

(iii) additional gerbils on a 24-h basis at 28 weeks.

Figure 6.5 presents the mean total aMT6s excreted by gerbils from the HF and LF groups at 7, 9, 11½, 16 and 28 weeks as bar charts with vertical bars above the columns representing SEM and asterisks represent the levels of statistical significance. At 7 weeks, the data from Section 6.1 were included because no additional data were obtained at this age.

Table 6.3 Summary of mean urinary aMT6s levels excreted per 24 hours from all gerbils (including the monitored groups of gerbils) at 7, 9, 11½, 16 and 28 weeks of age. Data expressed as mean ± SD, n = 8 - 30.

	AGE weeks	LOW-FLUORIDE FEMALES		HIGH-FLUORIDE FEMALES		LOW-FLUORIDE MALES		HIGH-FLUORIDE MALES	
		MEAN ± SD	n	MEAN ± SD	n	MEAN ± SD	n	MEAN ± SD	n
mean aMT6s ng/24-h	7	26.8 ± 6.8	12	18.1 ± 5.5	12	30.7 ± 7.9	12	16.4 ± 4.2	12
	9	25.6 ± 6.3	12	20.2 ± 6.2	12	27.9 ± 7.7	12	21.3 ± 5.1	20
	11½	26.5 ± 6.7	30	24.4 ± 7.6	24	33.0 ± 9.8	12	21.7 ± 6.5	21
	16	25.2 ± 9.5	18	23.5 ± 10.4	16	28.1 ± 13.1	21	30.4 ± 8.9	20
	28	17.4 ± 4.4	14	21.7 ± 5.7	13	24.4 ± 4.7	9	27.8 ± 7.5	8
mean aMT6s pg/g BW/24-h	7	602 ± 168	12	359 ± 109	12	569 ± 148	12	308 ± 76	12
	9	473 ± 107	12	365 ± 106	12	425 ± 113	12	348 ± 85	20
	11½	450 ± 117	30	390 ± 114	24	449 ± 111	12	310 ± 86	21
	16	380 ± 139	18	340 ± 134	16	342 ± 132	21	393 ± 94	20
	28	248 ± 58	14	289 ± 83	13	273 ± 102	9	306 ± 86	8
body weight g	7	45 ± 3.0	12	51 ± 5.6	12	54 ± 3.0	12	54 ± 5.8	12
	9	54 ± 3.4	12	55 ± 4.0	12	64 ± 5.1	19	61 ± 4.7	20
	11½	59 ± 4.7	30	63 ± 7.2	24	73 ± 6.9	12	70 ± 5.4	21
	16	66 ± 6.1	18	68 ± 9.1	16	80 ± 9.0	21	77 ± 6.4	20
	28	70 ± 4.4	14	76 ± 6.2	13	95 ± 18.1	9	91 ± 6.9	8

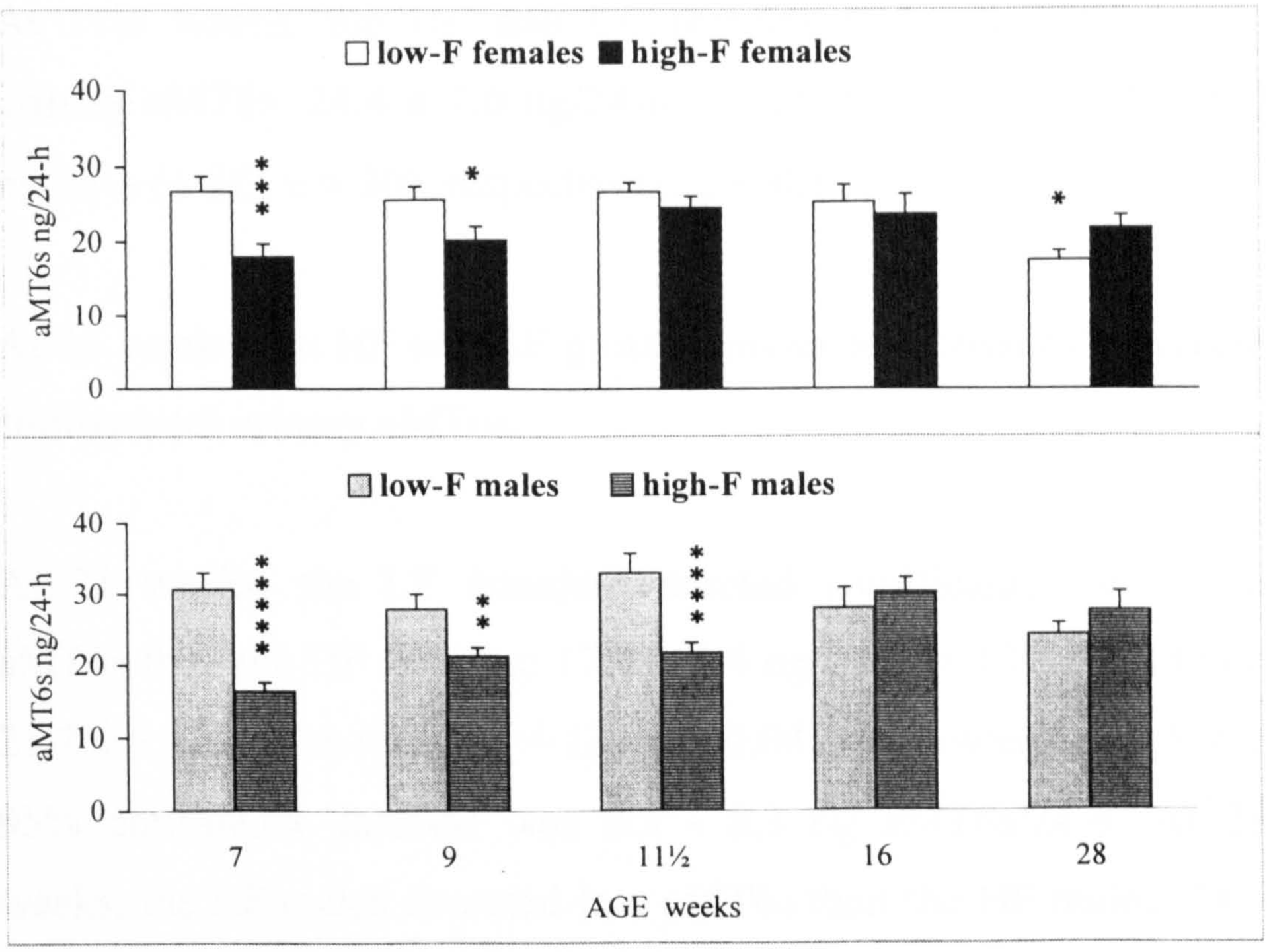

Fig. 6.5 A comparison of the mean absolute levels of aMT6s excreted by HF and LF gerbils at 7, 9, 11½, 16 and 28 weeks. LD: 12 12. Data expressed as mean ± SEM, n = 8-30; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0005$, *****$p < 0.00005$.

At 9 weeks, data from an additional group of HF males (n = 8) were pooled with data from Section 6.1. At 9 weeks, the HF males excreted significantly less aMT6s than the LF males: 21.3 ± 5.1 ng/24-h (± SD, n = 20) *vs.* 27.9 ± 7.7 ng/24-h (± SD, n = 12): $p < 0.01$. The power was 0.7; the 95% confidence interval was 2.0 to 11.2 ng aMT6s/24-h.

At 11½ weeks, the HF males excreted significantly less aMT6s than the LF males: 21.7 ± 6.6 ng/24-h (± SD, n = 21) *vs.* 33.0 ± 9.7 ng/24-h (± SD, n = 12), respectively: $p < 0.0004$. The power was

0.9; the 95% confidence interval was 5.5 to 17.1 ng aMT6s/24-h. At 11½ weeks, the HF and LF females excreted similar total urinary aMT6s: 24.4 ± 7.6 ng/24-h (± SD, n = 24) *vs.* 26.5 ± 6.7 ng/24-h (± SD, n = 30), respectively: $p < 0.3$.

At 16 weeks, the HF and LF groups (males and females) excreted similar total urinary aMT6s.

At 28 weeks, the LF females excreted significantly less total aMT6s than the HF females: 17.4 ± 4.4 ng/24-h (± SD, n = 14) *vs.* 21.7 ± 5.7 ng/24-h (± SD, n = 13): $p < 0.04$. The power was 0.5; the 95% confidence interval was 0.3 - 8.3 ng aMT6s/24-h. At 28 weeks, the LF males excreted less aMT6s than the HF males: 24.4 ± 4.7 ng/24-h (± SD, n = 9) *vs.* 27.8 ± 7.5 ng/24-h (± SD, n = 8), respectively but this difference was not significant: $p < 0.3$.

Figure 6.6 compares the mean absolute total aMT6s excreted by males and females from the same group at 7, 9, 11½, 16 and 28 weeks. The bar charts clearly show that the LF group excreted a constant rate of urinary aMT6s as they passed through puberty to adulthood. A significant difference was found between total aMT6s excreted by LF males and LF females at 7, 9, 11½, 16 and 28 weeks: $F = 3.19$, $df = 9, 142$, $p < 0.002$. The Student-Newman-Keuls test (SNK) isolated the 28-week-old LF females as being significantly different from the rest. The LF males, at 7, 9, 11½, 16 and 28 weeks, and LF females, at 7, 9, 11½ and 16 weeks, excreted similar total aMT6s: $F = 1.24$, $df = 8, 129$, $p < 0.3$.

At 28 weeks, the LF males excreted significantly more total aMT6s than the LF females: 24.4 ± 4.7 ng/24-h (± SD, n = 9) *vs*. 17.4 ± 4.4 ng/24-h (± SD, n = 14), respectively: $p < 0.002$. At 28 weeks, the LF females excreted significantly less aMT6s than at 16 weeks: 17.4 ± 4.4 ng/24-h, (± SD, n = 14) *vs.* 25.2 ± 9.5 ng/24-h, (± SD, n = 18) respectively: ($p < 0.01$). At 28 weeks, the LF males also excreted less aMT6s than at 16 weeks: 24.4 ± 4.7 ng/24-h (± SD, n = 9) *vs.* 28.1 ± 13.1 ng/24-h (± SD, n = 21) respectively; but this was not significant ($p < 0.4$).

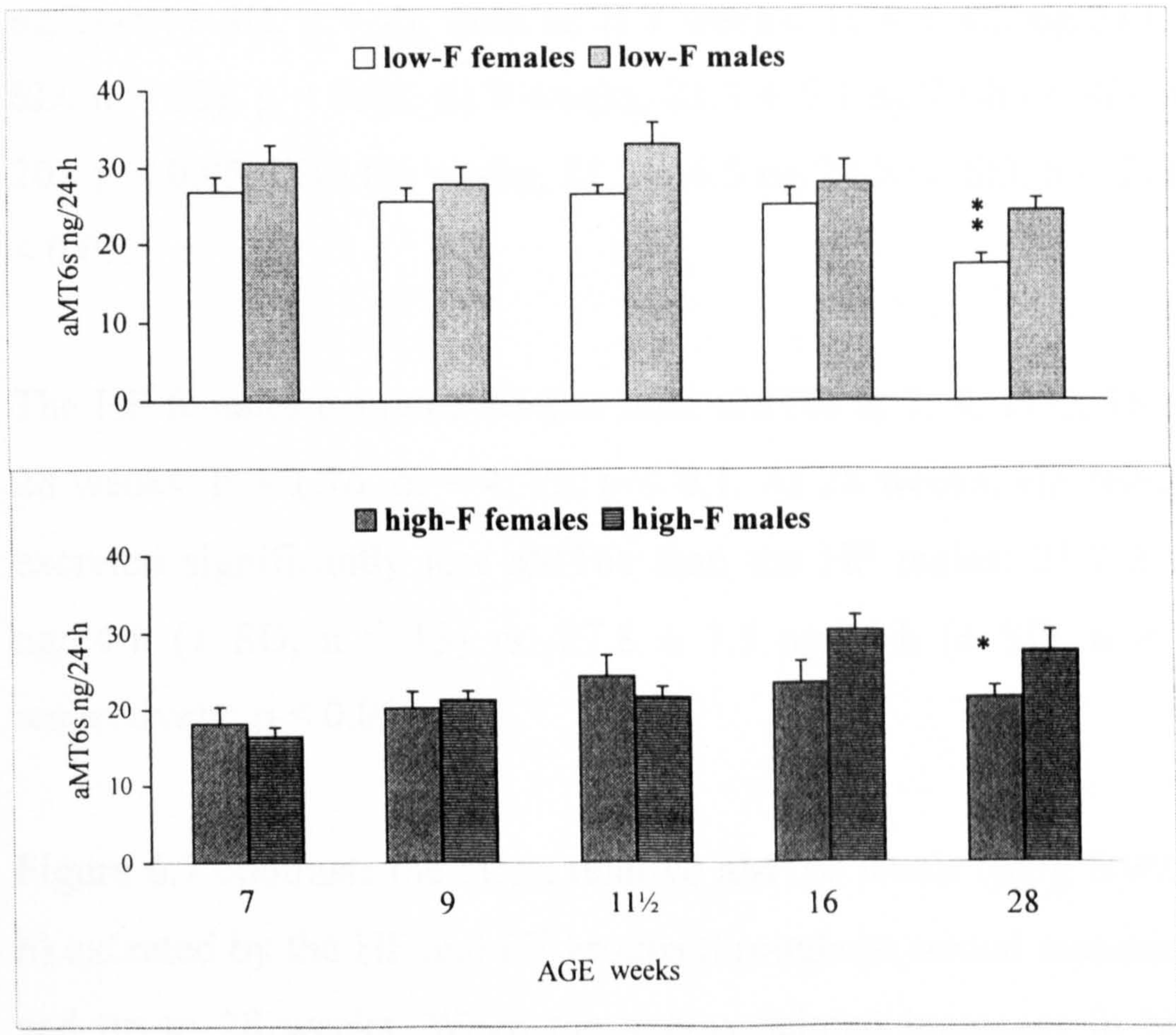

Fig. 6.6 A comparison of the mean absolute levels of urinary aMT6s excreted by male and female gerbils from the same group at 7, 9, 11½, 16 and 28 weeks under LD: 12 12. Data expressed as mean ± SEM, n = 8 - 30; * $p < 0.05$, ** $p < 0.002$

In contrast, the HF males and HF females excreted significantly different total aMT6s at 7, 9, 11½, 16 and 28 weeks: F = 5.11, df =

9, 148, $p < 0.00001$. Using SNK, at 16 weeks, the HF males excreted significantly more aMT6s, 30.4 ± 8.9 ng/24-h (± SD, n = 20), than at: i) 7 weeks, 16.4 ± 4.2 ng/24-h (± SD, n = 12): $p < 0.01$; ii) 9 weeks, 21.3 ± 5.1 ng/24-h (± SD, n =20): $p < 0.05$; iii) 11½ weeks, 21.7 ± 6.5 ng/24-h (± SD, n = 21): $p < 0.05$. At 16 weeks, the HF males excreted their highest levels of aMT6s, 30.4 ± 8.9 ng/24-h (± SD, n = 20); significantly more than at 11½ weeks: 21.7 ± 6.5 ng/24-h (± SD, n = 21): $p < 0.001$. Using SNK, at 28 weeks, the HF males excreted significantly more aMT6s, 27.8 ± 7.5 ng/24-h (± SD, n = 8), than at: i) 7 weeks, 16.4 ± 4.2 ng/24-h (± SD, n = 12): $p < 0.05$; ii) 9 weeks, 21.3 ± 5.1 ng/24-h (± SD, n = 20): $p < 0.05$; iii) 11½ weeks, 21.7 ± 6.5 ng/24-h (± SD, n = 21): $p < 0.05$.

The HF females excreted similar total aMT6s at 7, 9, 11½, 16 and 28 weeks: $F = 1.76$, df = 4, 72, $p < 0.1$. At 28 weeks, HF females excreted significantly less aMT6s than the HF males: 21.7 ± 5.7 ng/24-h (± SD, n = 13) *vs.* 27.8 ± 7.5 ng/24-h (± SD, n = 8), respectively: $p < 0.05$.

Figure 6.7 contrasts the mean relative aMT6s levels (pg/g BW/24-h) excreted by the HF and LF groups throughout sexual maturation and up to 28 weeks. When the age-associated increases in body weights were taken into consideration, the HF and LF groups excreted significantly different total aMT6s. The data at 7 weeks are taken from Section 6.1, i.e., the HF group excreted about half the total aMT6s as the LF group. At 9 weeks, (including data from eight additional HF males), the HF males excreted significantly less aMT6s than the LF males: 348 ± 85 pg/g BW/24-h (± SD, n = 20)

vs. 425 ± 113 pg/g BW/24-h, (± SD, n = 12): $p < 0.04$. The power was 0.5; the 95% confidence interval was 5-149 pg aMT6s/g BW/24-h.

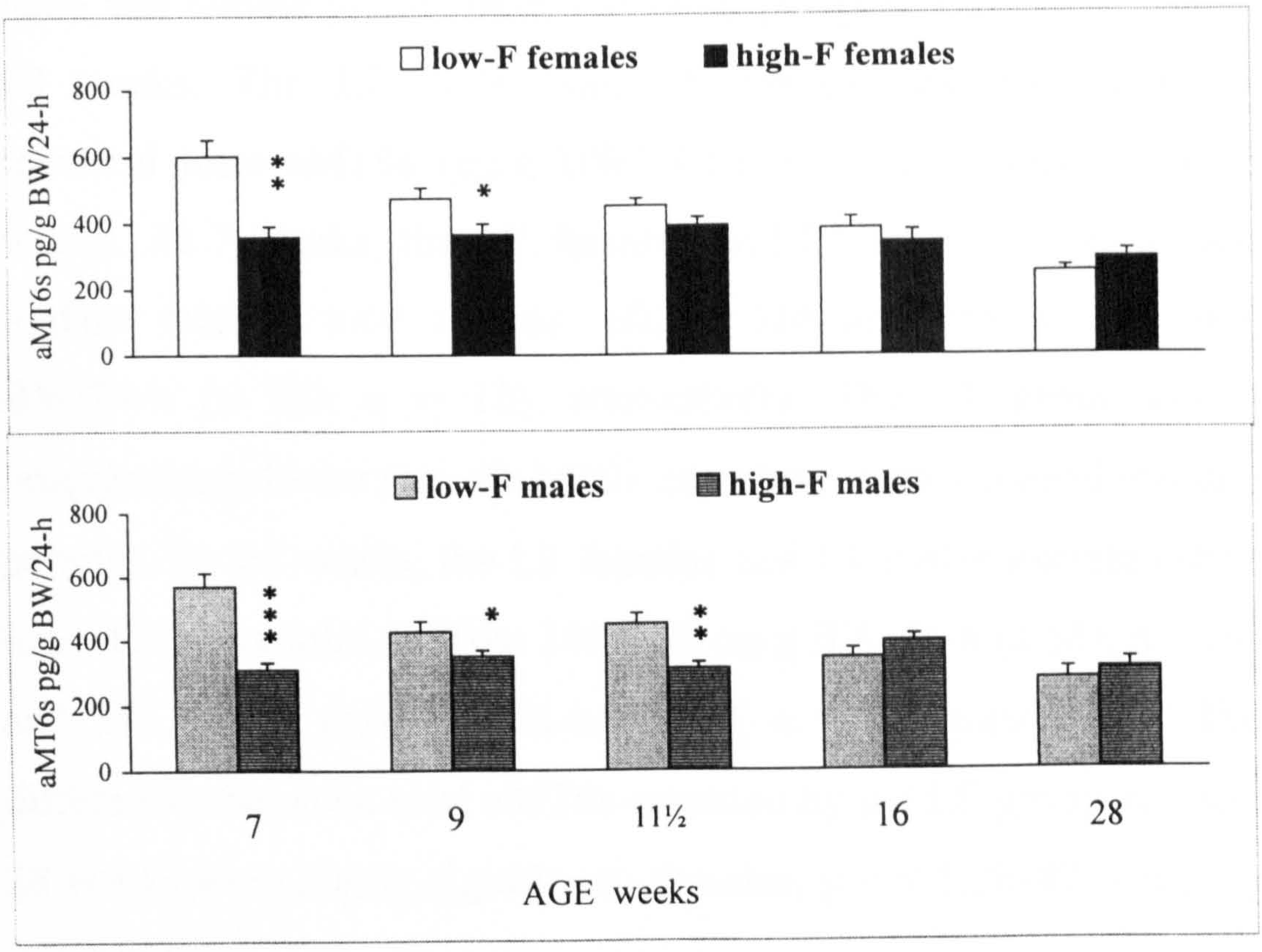

Fig. 6.7 A comparison of the relative levels of aMT6s excreted by gerbils from the HF and LF groups at 7, 9, 11½, 16 and 28 weeks. LD: 12 12, n = 8 - 30, *p < 0.05, **p < 0.0005, ***p < 0.00005.

At 11½ weeks, the HF males excreted significantly less aMT6s than the LF males: 310 ± 86 pg/g BW/24-h (± SD, n = 21) *vs.* 449 ± 111 pg/g BW/24-h (± SD, n = 12): p = < 0.0004. The power was 0.98; the 95% confidence interval was 68 to 210 pg aMT6s/g BW/24-h. At 11½ weeks, the HF females excreted less aMT6s than the LF females: 390 ± 114 pg/g BW/24-h (± SD, n = 24) *vs.* 450 ± 117 pg/g BW/24-h (± SD, n = 30): p = < 0.06. The power was 0.4; the 95% confidence interval was -4 to 124 pg aMT6s/g BW/24-h.

At 16 and 28 weeks, all gerbils excreted similar total aMT6s, in terms of body weight, irrespective of F-intake or gender.

Figure 6.8 compares the mean relative total aMT6s excreted by male and female gerbils from the same group at 7, 9, 11½, 16 and 28 weeks. The LF males and LF females excreted virtually identical total aMT6s (pg/g BW/24-h) throughout puberty to 28 weeks. At 7 weeks, the LF females and LF males excreted their highest relative total aMT6s: 602 ± 168 and 569 ± 148 pg/g BW/24-h (± SD, n = 12), respectively. The LF group had a progressively lower rate of aMT6s excretion as they passed through puberty. By 28 weeks, the LF females and LF males excreted their lowest relative total aMT6s: 248 ± 58 pg/g BW/24-h (± SD, n = 14) and 273 ± 102 pg/g BW/24-h (± SD, n = 9), respectively. The differences between total aMT6s excreted by the LF group at 7 and 28 weeks were highly significant: females, $p = < 1.2E-07$; males, $p = < 0.0001$.

During the time of sexual maturation, the HF group excreted a different pattern of urinary aMT6s than the LF group. Taking body weights into consideration, the HF females excreted similar total aMT6s at 7, 9, 11½, 16 and 28 weeks: $F = 1.8$, $df = 4, 72$, $p = < 0.1$. For example, the HF females excreted 359 ± 109 pg aMT6s/g BW/24-h (± SD, n = 12) at 7 weeks and 289 ± 83 pg aMT6s/g BW/24-h (± SD, n = 12) at 28 weeks: $p = < 0.1$.

At 7, 9 and 11½ weeks, the HF males and HF females had similar rates of aMT6s excretion: $F = 2.02$, $df = 5, 95$, $p < 0.08$. $F = 1.32$, $df = 2, 50$, $p < 0.3$. At 11½ weeks, the HF males excreted significantly

less aMT6s than the HF females: 310 ± 86 pg/g BW/24-h (± SD, n = 21) *vs.* 390 ± 114 pg/g BW/24-h, (± SD, n = 24), respectively: F = 6.85, df = 1, 43, $p < 0.01$. At 16 and 28 weeks, the HF males and HF females excreted similar total aMT6s when their body weights were taken into consideration.

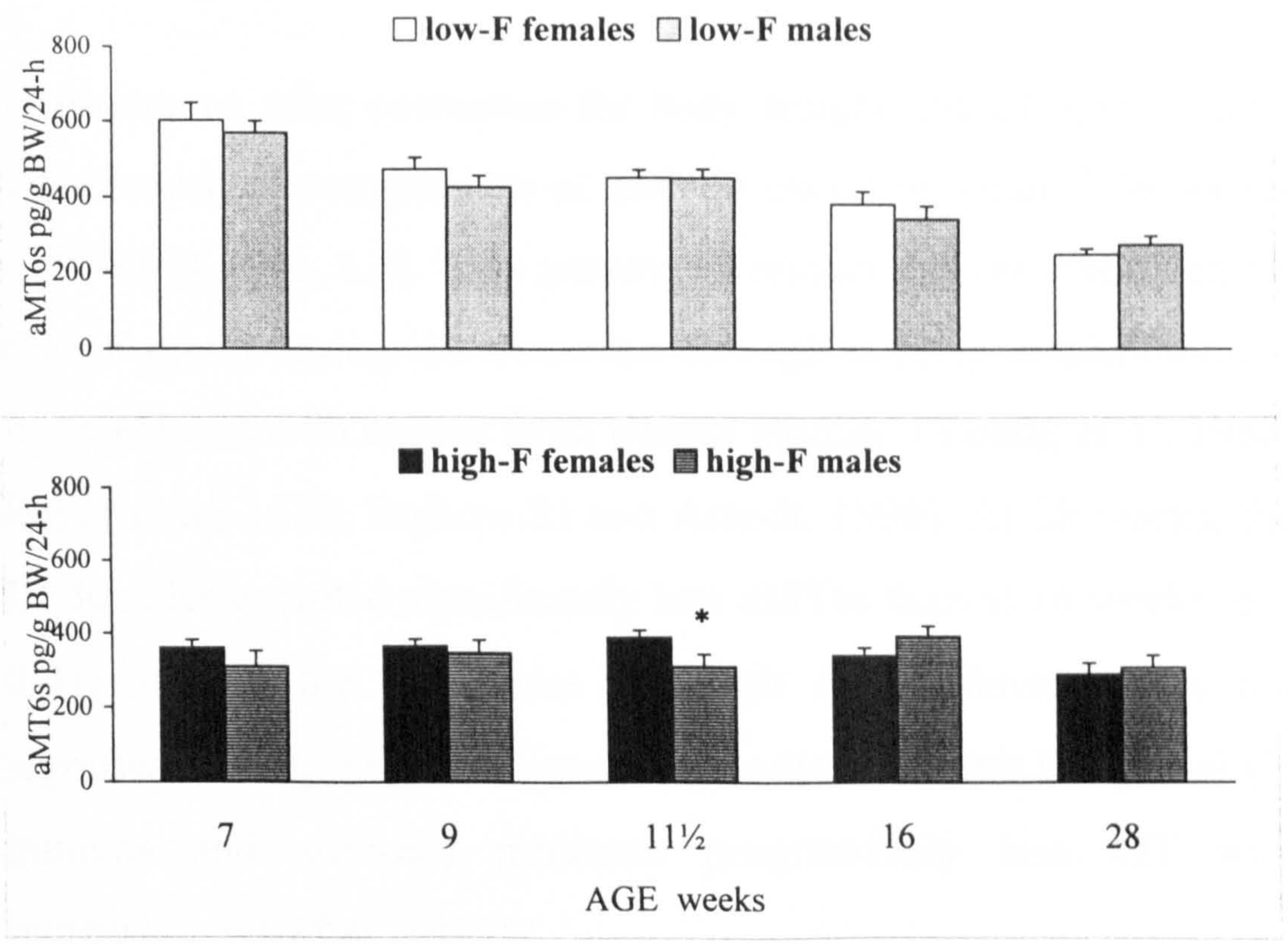

Fig. 6.8 A comparison of the relative levels of urinary aMT6s excreted by male and female gerbils from the same group at 7, 9, 11½, 16 and 28 weeks; LD: 12 12. Data expressed as mean ± SEM, * $p < 0.01$.

6.3 Conclusions

To the best of my knowledge, this is the first longitudinal study of urinary aMT6s excretion during the sexual maturation of the gerbil.

Prepubescent, pubertal and young adult male and female gerbils in the LF group excreted similar, constant total aMT6s from 7-11½ weeks. This result is consistent with previous human studies which used rates of urinary metabolite excretion to investigate the changes in pineal MT production during sexual maturation (Young *et al*, 1988; Bojkowski and Arendt, 1990; Tetsuo *et al*, 1982; Sizonenko *et al*, 1985).

Furthermore, after correction for body weight, the LF group had a significantly decreased rate of aMT6s excretion from 7-16 weeks ($p < 0.01$). (Fig. 6.4). This pattern of urinary aMT6s excretion by the LF group during the transition through puberty to adulthood is in agreement with results from human studies: (Young *et al*, 1988; Rager *et al*, 1989; Bojkowski and Arendt, 1990). At 28 weeks, the LF females excreted significantly less aMT6s than at 16 weeks: $p < 0.01$; so did the LF males although the difference was not significant. (Fig 6.6). It is generally understood that the pineal (in humans and animals) produces progressively less MT with increasing age after puberty.

After correction for body weight, the LF males and LF females excreted similar total aMT6s and in an almost identical pattern throughout the experiment. (Fig. 6.8). There was no sex difference in the total aMT6s excreted by the LF group through puberty to adulthood. This observation is consistent with human studies which found no sex difference in the excretion rate of urinary aMT6s during normal puberty (Young *et al*, 1988; Bojkowski and Arendt, 1990); or nocturnal plasma MT levels (Waldhauser *et al*, 1984; 1988). On the other hand, at 3 weeks, 2 and 8 months, male rats

had significantly higher total aMT6s than female rats (Yie *et al*, 1992).

The data on rates of urinary aMT6s excretion by the LF group during puberty were predictable and consistent with results from other species (including humans). Therefore, the LF group exemplifies the normal pattern of aMT6s excretion by gerbils during pubertal development (under LD: 12 12). This fact accentuates the significance of the conflicting results obtained from the HF group.

During pubertal development, the HF group excreted far less aMT6s than the LF group. The level of significance between the excretion rates of aMT6s by the groups was highest in the prepubescent gerbils. At 7 weeks, the HF males excreted about half as much total aMT6s as the LF males. (Table 6.1). The HF males continued to excrete significantly less aMT6s than the LF males throughout pubertal development: at 9 weeks, $p < 0.005$; and, at 11½ weeks, $p < 0.001$. By 16 weeks, the HF males excreted similar aMT6s levels as the LF males. In fact, all gerbils excreted similar total aMT6s at 16 weeks: between 340-393 pg/g BW/24-h.

At 7 weeks, the HF females also excreted almost half as much aMT6s as the LF females:. 359 ± 109 *vs*. 602 ± 168 pg/g BW/24-h. (The data are expressed in relative terms because, at 7 weeks, the HF females were significantly heavier than the LF females). Thereafter, the significance between the HF and LF females diminished: by 9 weeks, $p < 0.05$; and by 11½ weeks, the HF females and LF females excreted similar total aMT6s: 390 ± 114

pg/g BW/24-h (± SD, n = 24) and 450 ± 117 pg/g BW/24-h (± SD, n = 30), respectively. In addition, at 11½ weeks, the HF females excreted similar total aMT6s as the LF males: 390 ± 114 pg/g BW/24-h (± SD, n = 24) and 449 ± 111 pg/g BW/24-h (± SD, n = 12), respectively. (Table 6.3). At 11½ weeks, the HF females excreted significantly more aMT6s ($p < 0.02$) than the HF males (after correction for body weight). Therefore, unlike the LF group, there was a sex difference between the relative excretion rates of aMT6s in the HF group. By 11½ weeks, the HF female gerbils presumably had a normal rate of urinary aMT6s excretion. When body weights were considered, the HF group had significantly different rates of aMT6s excretion with increasing age compared to the LF group. The HF females had a constant relative rate of aMT6s excretion from 7 to 28 weeks. The HF males had a constant relative rate of aMT6s excretion from 7 to 11½ weeks. Figure 6.8 clearly illustrates the different patterns of excretion of aMT6s (pg/g BW) throughout the experiment: a uniform, constant excretion of aMT6s by the HF group *vs.* a significant, progressive decrease in aMT6s excretion by the LF group.

The urinary aMT6s levels are a valid index of pineal MT production in the gerbil. (Chapter 5). Therefore, the data can be re-evaluated. In absolute terms, the LF group had a constant pineal MT output from 7 through to 28 weeks. When body weights are considered, the pineal MT output in the LF group progressively declined with age until adulthood. There is no sex difference. In contrast, the prepubescent HF gerbils had significantly lower pineal MT outputs than the LF group. When body weights are considered, the pineal MT output in the HF group remained constant (albeit at a

significantly lower level than the LF group) until 11½ weeks in the HF females; until 16 weeks in the HF males. The inhibitory effects of F on pineal MT output cease when gerbils reach adulthood.

The most plausible explanation for the significantly reduced excretion rate of aMT6s by the sexually immature HF gerbils is that F inhibits the synthesis of pineal MT. One can only speculate whereabouts in the pathway from tryptophan to MT this inhibition occurs. There may be a general inhibition of the enzymes involved in the formation of MT: particularly enzymic reactions requiring divalent cations. Several calcium-dependent reactions are involved in the biosynthesis and release of MT. By binding with calcium, F may inhibit the biosynthesis of MT or a MT precursor, e.g., serotonin. Fluoride may cause a diminished uptake of tryptophan by the pinealocytes. Enamel fluorosis may be caused by the inhibitory effects of F on the uptake of the amino acids necessary for protein synthesis in the rat ameloblast (serine and proline) (Kruger, 1970; 1972). Fluoride may affect the synthesis of other pineal products, besides melatonin.

Alternatively, F may affect the rate of MT metabolism in the liver or the clearance rate of aMT6s in the kidney with subsequent alterations in the levels of urinary aMT6s. This possibility is discussed in Section 7.2.

CHAPTER 7 - Effects of Fluoride on the Circadian Rhythm of Urinary aMT6s in Gerbils Monitored at 11½ and 16 Weeks of Age

7.1 Results

The intra-assay CV% for the RIAs for urinary aMT6s were 4.3%, 8.2% and 5.7% at 3.5, 12.3 and 24.3 ng/ml respectively. See table C.1 in Appendix C. The inter-assay CV% for RIAs for urinary aMT6s levels excreted by gerbils at 11½ and 16 weeks were 4.5%, 5.2% and 9.2% at 3.7, 13.7 and 23 ng/ml respectively. See table C.4.

7.1.1 Circadian Profiles of aMT6s in Gerbil Urine at 11½ Weeks

At 11½ weeks, five gerbils excreted substantially higher levels of urinary aMT6s in day 1 than in day 2, i.e., CV% between the total aMT6s excreted in day 1 and day 2 was greater than 10%. See table C.8 in Appendix C. In these 5 instances, the circadian data during day 2 were ignored and replaced with data from day 1.

Table 7.1 presents the 48-h profiles of urinary aMT6s in male and female gerbils aged 11½ weeks from HF and LF groups (n = 11-12) kept in LD: 12 12. Values are expressed as ng aMT6s/3-h and pg aMT6s/g BW/3-h and represent the mean ± SD, n = 11-12.

Table 7.1 Summary of mean circadian profiles of aMT6s as ng/3-h and pg/g BW/3-h over 48-hours in male and female gerbils aged 11½ weeks from high-F and low-F groups; data expressed as means ± SD, n = 12.

TIME	aMT6s ng/3-hours				aMT6s pg/g BW/3-hours			
	11½ week low-F females	11½ week high-F females	11½ week low-F males	11½ week high-F males	11½ week low-F females	11½ week high-F females	11½ week low-F males	11½ week high-F males
	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD
1300 - 1600	1.6 ± 1.0	1.2 ± 0.5	1.8 ± 1.0	1.1 ± 0.5	26 ± 15	19 ± 7	25 ± 12	15 ± 8
1600 - 1900	1.6 ± 0.9	1.9 ± 0.7	1.6 ± 1.2	1.3 ± 0.6	27 ± 16	30 ± 12	23 ± 17	17 ± 8
1900 - 2200	1.7 ± 0.7	1.7 ± 0.6	2.2 ± 0.9	2.2 ± 1.1	28 ± 12	27 ± 7	29 ± 12	30 ± 14
2200 - 0100	2.9 ± 1.6	4.3 ± 3.1	3.6 ± 2.3	3.2 ± 1.7	49 ± 28	67 ± 48	48 ± 26	44 ± 23
0100 - 0400	6.9 ± 4.8	7.2 ± 4.8	7.8 ± 5.1	4.6 ± 2.2	117 ± 79	111 ± 73	104 ± 65	63 ± 28
0400 - 0700	7.2 ± 3.9	5.0 ± 2.1	8.6 ± 2.9	3.5 ± 1.5	119 ± 61	79 ± 32	120 ± 47	48 ± 21
0700 - 1000	3.5 ± 2.0	2.4 ± 1.1	4.5 ± 3.1	2.7 ± 1.3	59 ± 36	38 ± 18	61 ± 39	37 ± 18
1000 - 1300	1.7 ± 0.9	1.9 ± 0.8	2.3 ± 1.1	2.1 ± 1.2	29 ± 17	29 ± 11	31 ± 12	28 ± 16
1300 - 1600	2.0 ± 0.9	1.8 ± 1.0	2.6 ± 0.9	1.2 ± 0.7	34 ± 17	29 ± 14	36 ± 12	17 ± 9
1600 - 1900	2.4 ± 1.6	2.8 ± 1.3	2.8 ± 1.2	1.5 ± 1.0	39 ± 23	44 ± 20	38 ± 16	21 ± 12
1900 - 2200	2.2 ± 0.9	1.8 ± 0.9	2.5 ± 1.0	2.0 ± 0.6	37 ± 14	28 ± 13	34 ± 12	28 ± 7
2200 - 0100	3.0 ± 1.8	3.2 ± 2.8	2.8 ± 1.5	3.7 ± 2.2	51 ± 29	51 ± 44	38 ± 22	50 ± 30
0100 - 0400	6.3 ± 3.8	6.7 ± 4.8	7.8 ± 4.6	4.7 ± 1.7	103 ± 57	103 ± 74	106 ± 61	64 ± 22
0400 - 0700	6.9 ± 3.0	5.8 ± 3.9	9.0 ± 4.8	5.5 ± 2.5	115 ± 48	91 ± 57	122 ± 57	75 ± 34
0700 - 1000	3.8 ± 2.2	2.7 ± 1.1	3.8 ± 1.8	2.6 ± 1.6	67 ± 43	42 ± 14	52 ± 23	35 ± 20
1000 - 1300	2.3 ± 1.0	2.4 ± 1.3	2.2 ± 0.9	2.2 ± 1.2	40 ± 16	38 ± 19	30 ± 10	30 ± 16
total aMT6s/48-h	55.8 ± 13.9	52.8 ± 19.7	65.9 ± 19.5	44.0 ± 11.3	940 ± 200	824 ± 279	898 ± 223	600 ± 147
total aMT6s/day 1	27.0 ± 7.2	25.5 ± 9.6	32.5 ± 9.9	20.6 ± 5.3	454 ± 103	399 ± 137	442 ± 115	282 ± 71
total aMT6s/day 2	28.8 ± 6.9	27.3 ± 10.6	33.5 ± 9.8	23.3 ± 6.4	485 ± 101	425 ± 150	456 ± 112	318 ± 81
mean aMT6s/24-h	27.9 ± 6.9	26.4 ± 9.9	33.0 ± 9.8	22.0 ± 5.7	470 ± 100	412 ± 140	449 ± 112	300 ± 73
mean aMT6s pg/g BW/24-h	470 ± 100	412 ± 140	449 ± 112	300 ± 73				

N.B. Data excludes high-F female which died at 14 weeks. Day 1 circadian data were repeated in day 2 where there were substantial differences in the levels of aMT6s recorded in day 1 and day 2, i.e., CV% > 10% due to higher levels in day 1.

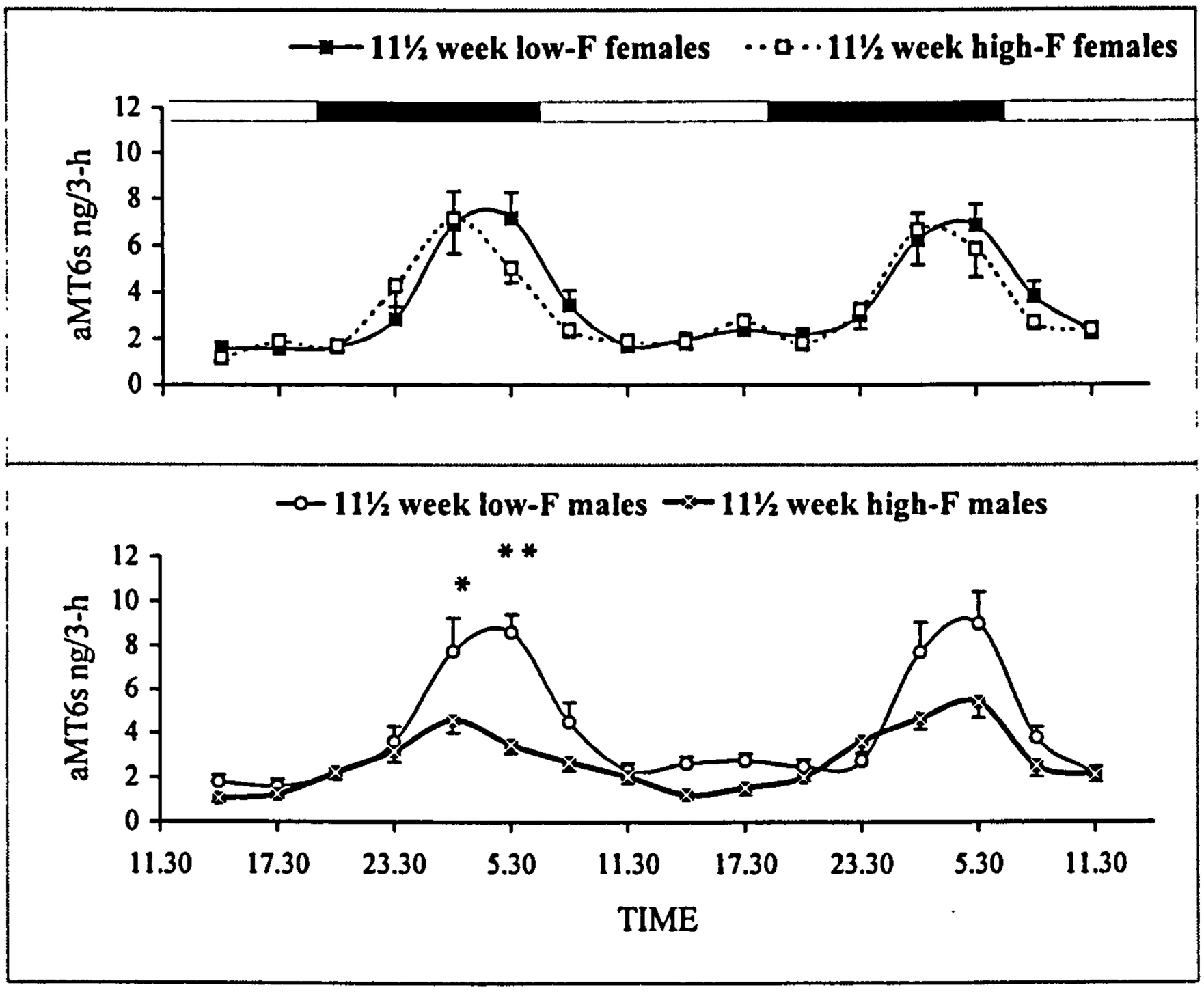

Fig. 7.1 Comparison of the absolute 48-h profiles of urinary aMT6s in gerbils from the HF and LF groups at 11½ weeks. LD: 12 12; black horizontal bars indicate periods of darkness; labels on the x-axis represent the mid-points of the 3-h intervals; data points represent mean ± SEM, n = 11 - 12; *p < 0.04, **p < 0.0002.

Figure 7.1 compares the mean 48-h profiles of urinary aMT6s in 11½-week-old male and female gerbils from the HF and LF groups, under LD: 12 12. The black horizontal bars represent the dark periods, the vertical bars above (or below) the data points represent the SEM, and asterisks represent the level of statistical significance. All gerbils had well-defined circadian rhythms of urinary aMT6s although the 11½-week-old HF male produced the least robust rhythm. The levels of urinary aMT6s began to rise in the second 3-h interval of the dark period; peaked between 0100 and 0700 and then declined to daytime levels after 1000.

Figure 7.1 clearly shows that the LF males excreted significantly higher mean values of aMT6s during the night than the HF males. During the intervals, 0100 to 0400, the LF males excreted significantly more aMT6s than the HF males: 7.8 ± 4.8 *vs.* 4.7 ± 1.9 ng/3-h (± SD): $p < 0.04$. During the intervals, 0400 to 0700, the LF males excreted almost twice as much aMT6s as the HF males: 8.8 ± 3.9 *vs.* 4.5 ± 2.3 ng/3-h (± SD) respectively: $p < 0.0002$. During the 12-h night-time intervals, 2200 to 1000, the LF males excreted significantly more urinary aMT6s than the HF males: 24.0 ± 8.7 *vs.* 15.2 ± 4.1 ng/12-h (± SD) respectively: $p < 0.005$.

At 11½ weeks, the HF and LF females had similar 48-h profiles of urinary aMT6s. They excreted 18.7 ± 8.9 and 20.2 ± 5.4 ng/12-h (± SD) respectively, during the 12-h night-time intervals, 2200 to 1000. The HF group (particularly the males) showed a shift of average dark-phase peak of aMT6s towards the left.

All gerbils, irrespective of gender or F-intake, excreted low levels of aMT6s during the day, i.e., about 2 ng/3-h. Urine excreted during the first daytime interval (0700 to 1000) still had elevated aMT6s values.

Figure 7.2 compares the relative 48-h profiles of urinary aMT6s in the HF and LF groups at 11½ weeks. When body weights were taken into consideration, the graphs show that: i) the HF males had a significantly different mean 48-h aMT6s profile than the LF males; ii) the HF and LF females had similar 48-h profiles. During the 12-h night-time periods, 2200 to 1000, the LF males excreted significantly more aMT6s than the HF males: 332 ± 105 *vs.* 208 ±

54 pg/g BW/12-h (± SD), respectively: $p < 0.002$. During the intervals, 0100 to 0400, the LF males excreted 107 ± 72 *vs.* 64 ± 25 pg aMT6s/g BW/3-h (± SD) by the HF males: $p < 0.008$. During the intervals, 0400 to 0700, the LF males excreted twice as much aMT6s as the HF males: 121 ± 51 *vs.* 61 ± 31 pg/g BW/3-h (± SD) respectively: $p < 0.00001$. In contrast, the 11½-week-old HF and LF females produced similar 48-h profiles of aMT6s and excreted similar amounts of aMT6s during the night-time periods, 2200 to 1000: i.e., 291 ± 132 *vs.* 340 ± 78 pg/g BW/12-h (± SD) respectively.

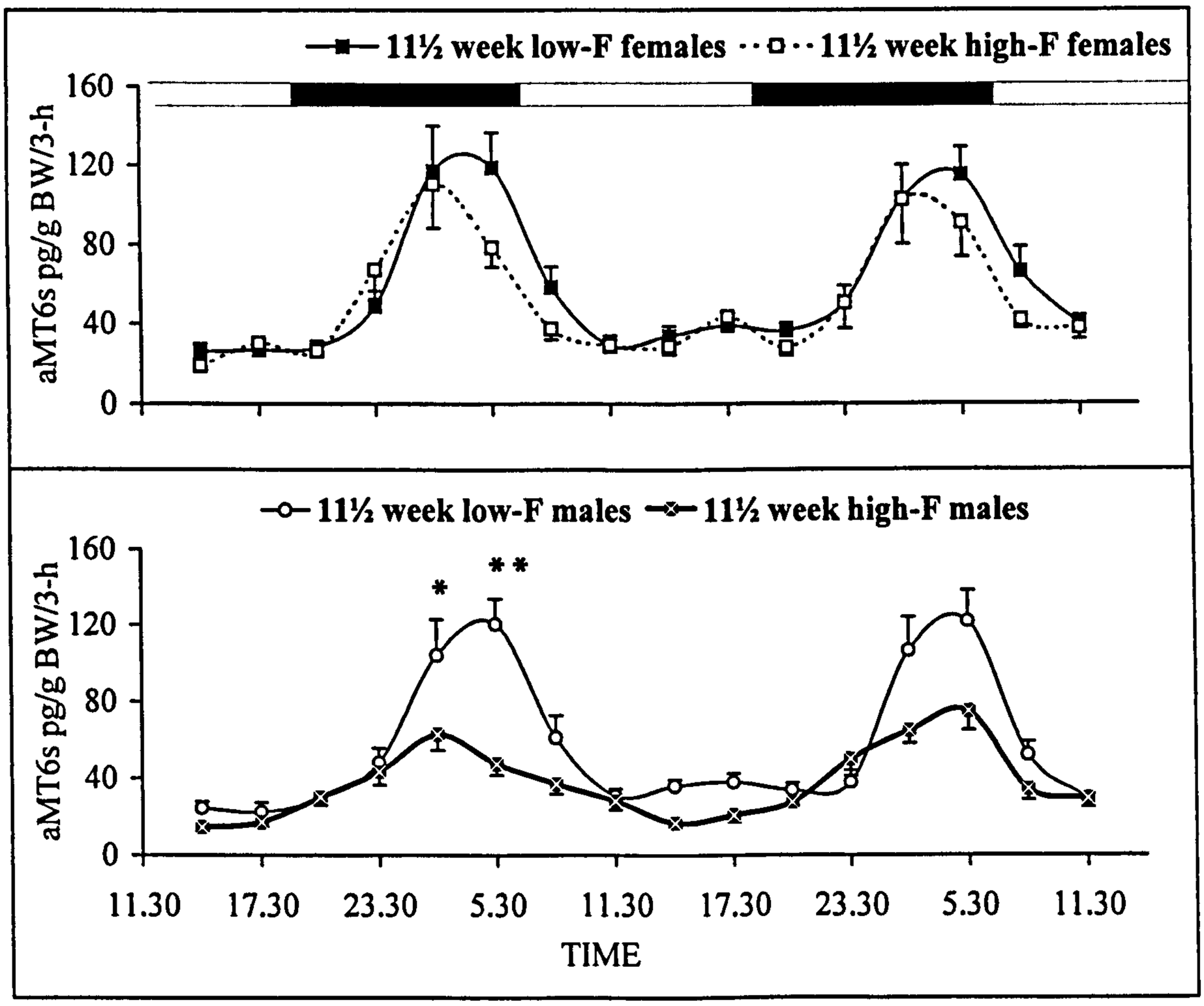

Fig. 7.2 Comparison of the relative 48-h profiles of urinary aMT6s in gerbils aged 11½ weeks from the HF and LF groups. LD: 12 12. Black horizontal bars represent periods of darkness; data points represent mean ± SEM, n = 11-12; labels on the x-axis represent the mid-points of the 3-h intervals; *$p < 0.01$, **$p < 0.00001$.

All 11½-week-old gerbils, irrespective of gender or F-intake, excreted 28 ± 15 pg aMT6s/g BW/3-h (± SD) during the daytime except during the first interval after lights-on (0700-1000).

Figure 7.3 illustrates that the 11½-week-old LF males and LF females produced similar 48-h profiles of urinary aMT6s. When the data were expressed as a function of body weights, their 48-h profiles were superimposable.

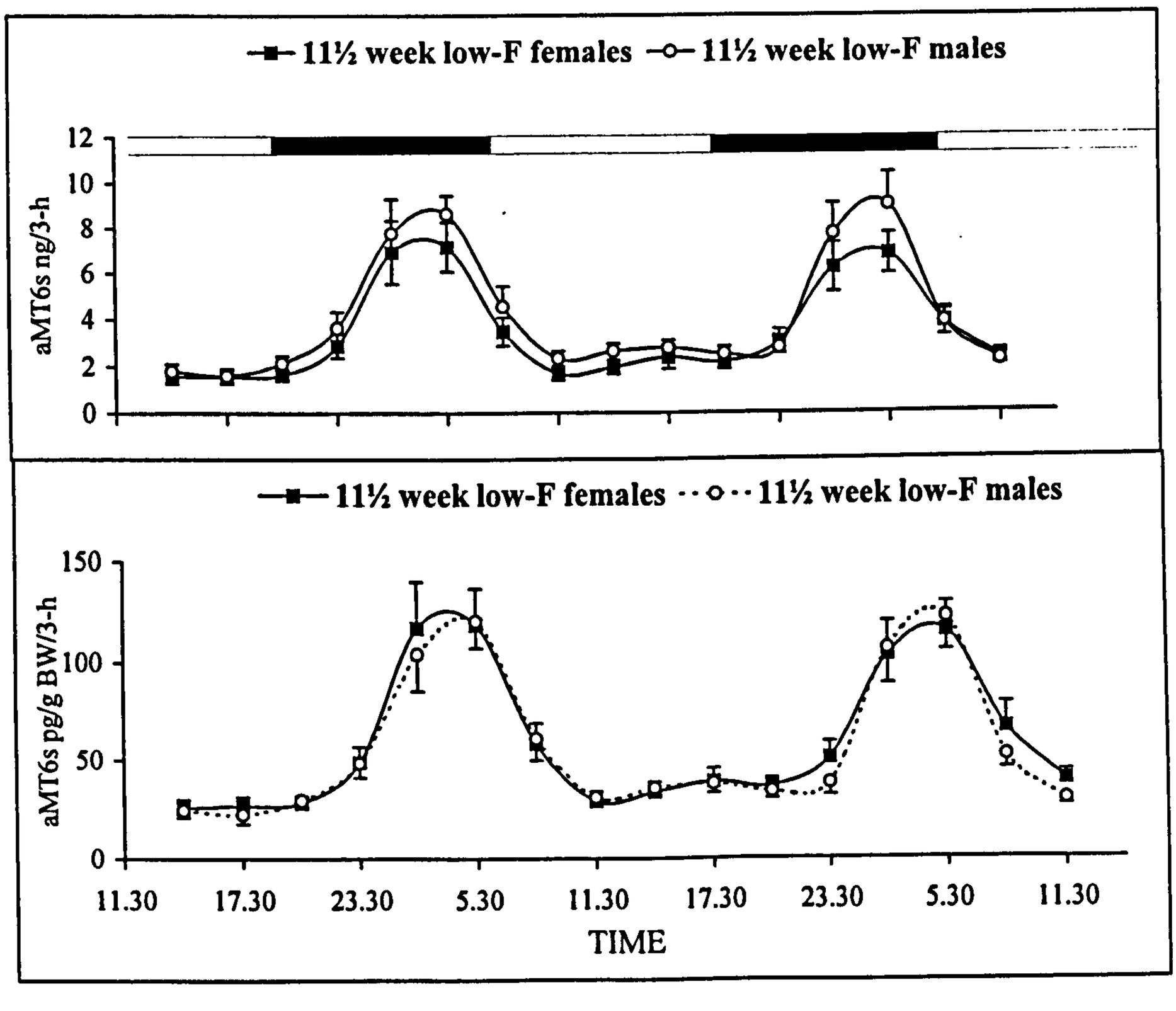

Fig. 7.3 Comparison of the absolute and relative 48-h profiles of urinary aMT6s in male and female gerbils from the LF group aged 11½ weeks. LD: 12 12. Black horizontal bars indicate periods of darkness; data points represent mean ± SEM, n = 12.

During the night-time, 2200 to 1000, the LF males and females excreted virtually identical amounts of urinary aMT6s: 326 ± 105

vs. 340 ± 78 pg/g BW/12-h (± SD) respectively. The LF males excreted higher nocturnal values of aMT6s (in absolute terms) than the LF females but the differences were not significant.

Figure 7.4 shows that, at 11½ weeks, the HF males and HF females had significantly different aMT6s profiles. Between 0100 to 0400, the HF females excreted significantly more aMT6s than the HF males: in absolute terms: 6.9 ± 4.7 *vs.* 4.7 ± 1.9 ng/3-h, respectively, $p < 0.04$; in relative terms, 107 ± 72 *vs.* 64 ± 25 pg/g BW/3-h (± SD), respectively: $p < 0.008$.

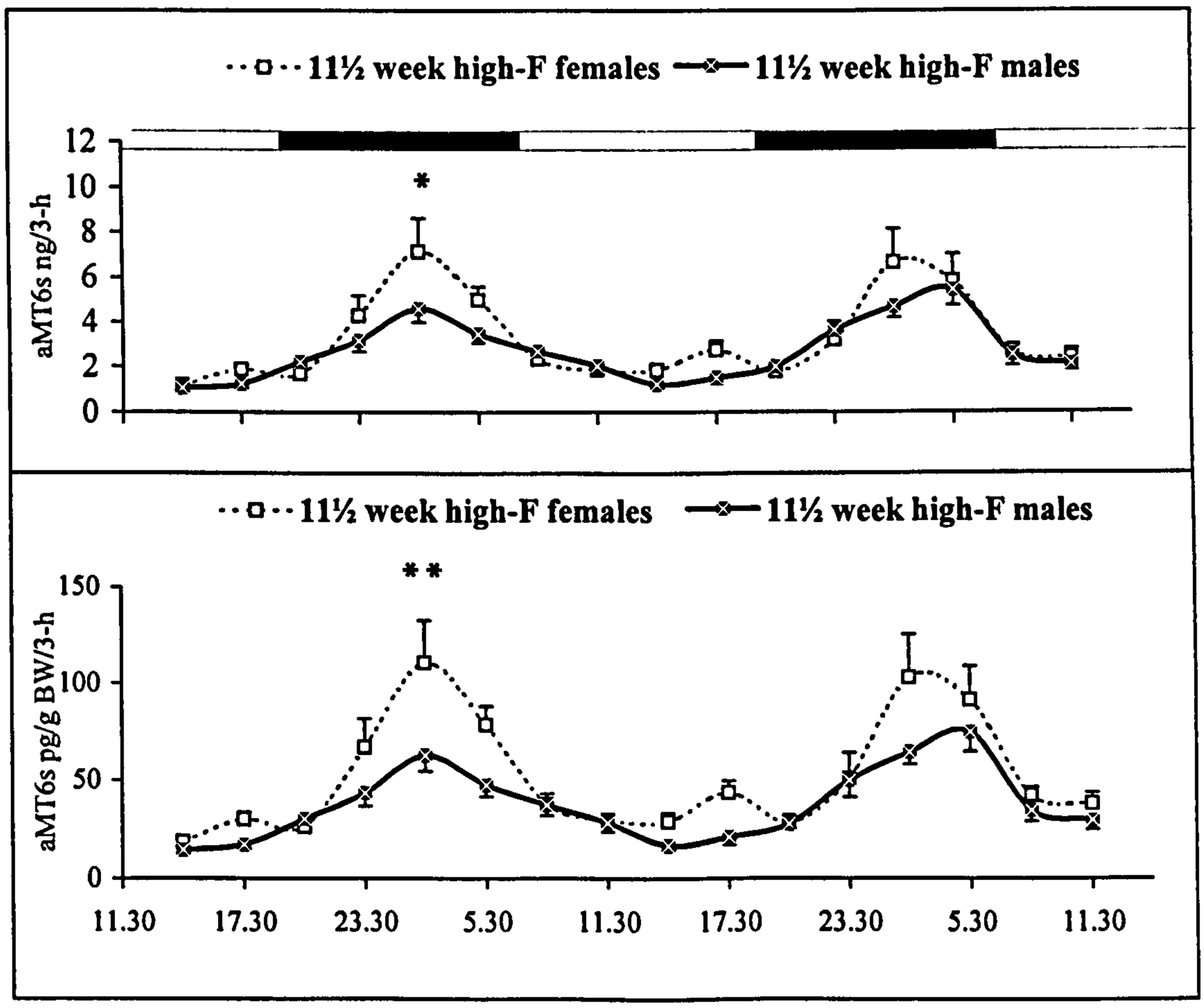

Fig. 7.4 Comparison of the absolute and relative 48-h profiles of aMT6s in HF males and HF females at 11½ weeks. LD: 12 12. Black bars indicate darkness; mean ± SEM, n = 11-12; *$p < 0.05$, **$p < 0.01$.

During the intervals, 0400 to 0700, the HF females excreted more aMT6s than the HF males: 5.4 ± 2.7 *vs.* 4.5 ± 1.8 ng/3-h (± SD); and in relative terms: 85 ± 39 *vs.* 61 ± 24 pg/g BW/3-h (± SD) respectively. These differences were not significant. During the night-time, (2200 to 1000), the HF females excreted more aMT6s than the HF males: 18.7 ± 9.0 *vs.* 15.2 ± 4.1 ng/12-h (± SD): $p < 0.3$; in relative terms: 291 ± 132 *vs.* 208 ± 54 pg/g BW/12-h respectively: $p < 0.07$. These differences were not significant.

Figure 7.5 illustrates the similarity between the relative 48-h profiles of urinary aMT6s in LF males, LF females and HF females at 11½ weeks.

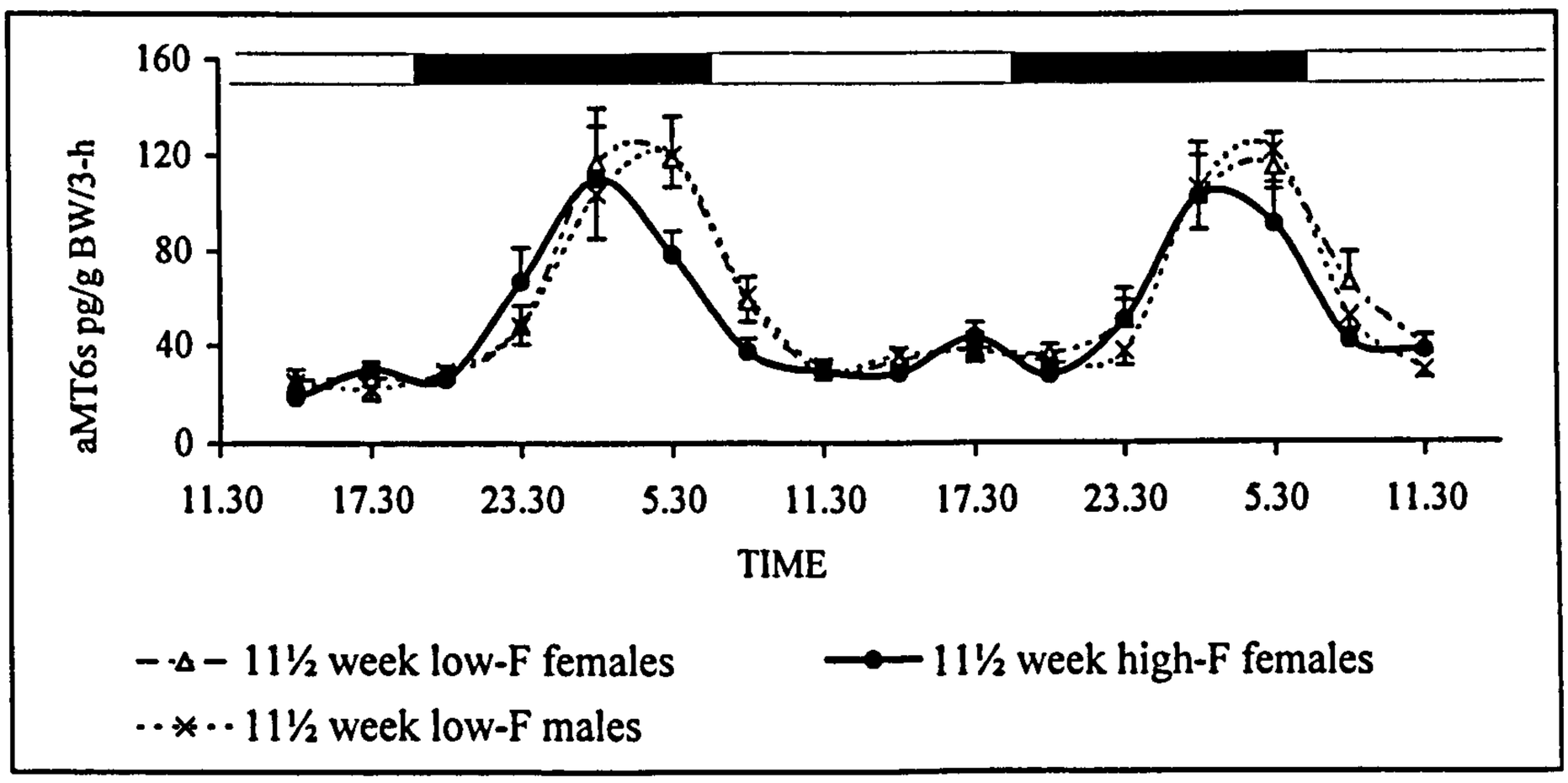

Fig. 7.5 Comparison of the relative 48-h profiles of urinary aMT6s in LF males, LF females and HF females at 11½ weeks. LD: 12 12; black bars indicate darkness; mean ± SEM, n = 11-12.

Figure 7.6 compares the absolute and relative profiles of aMT6s in 11½-week-old gerbils which urinated during every 3-h interval throughout 24-h. The graphs clearly show that the HF and LF females produced similar absolute and relative profiles of aMT6s; whereas the LF males excreted notably higher nocturnal values of

aMT6s than the HF males. The LF males and HF males excreted their highest peak values of aMT6s between 0400-0700, i.e., 9.8 ± 5.2 *vs.* 5.6 ± 3.0 ng/3-h or 134 ± 61 *vs.* 79 ± 43 pg/g BW/3-h (± SD), respectively. The LF females and HF females excreted their highest values of aMT6s earlier in the dark phase, i.e., between 0100-0400: 7.0 ± 3.7 *vs.* 6.8 ± 4.7 ng/3-h or 114 ± 58 *vs.* 104 ± 72 pg/g BW/3-h (± SD), respectively.

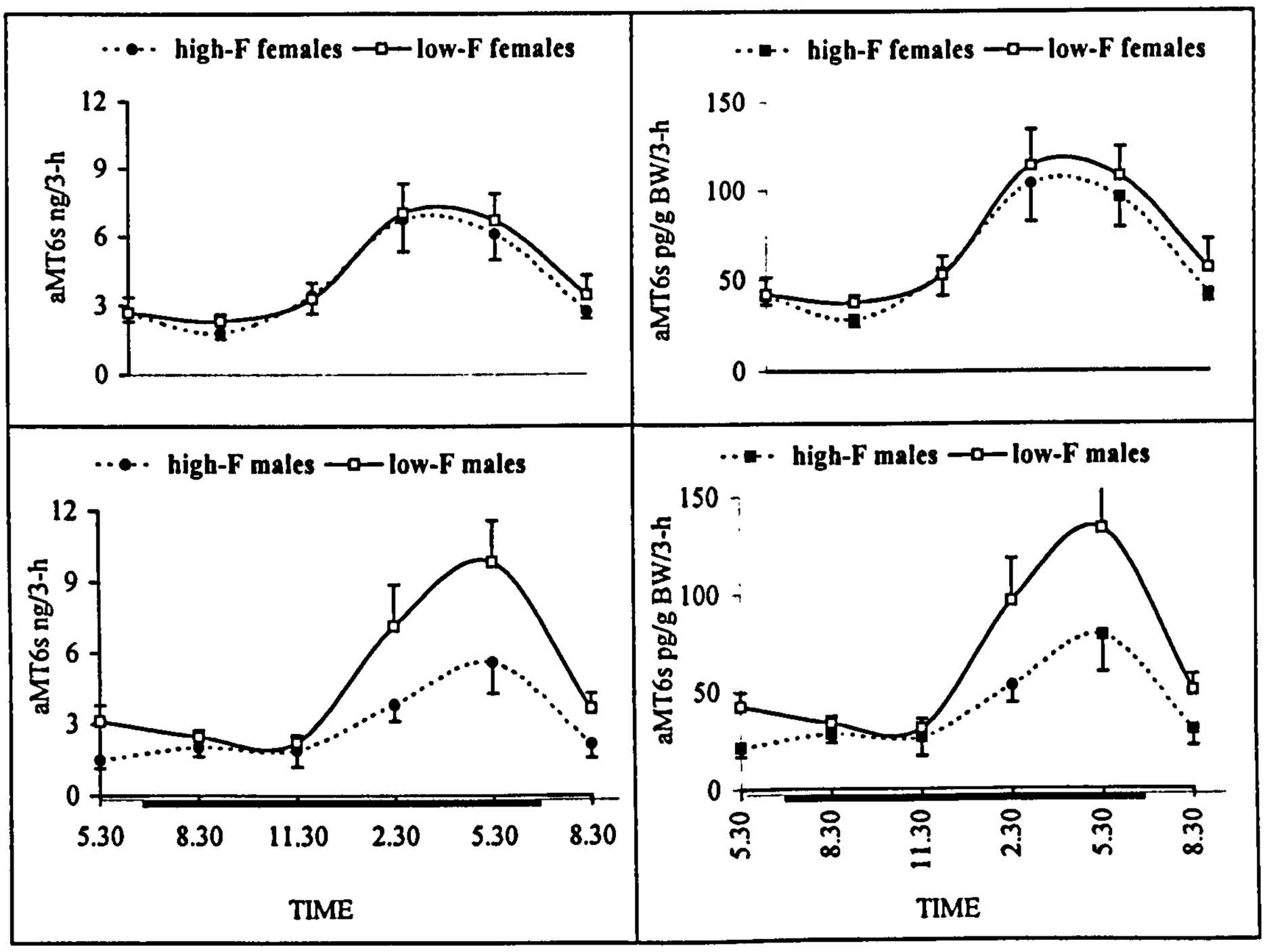

Fig. 7.6 Comparison of the mean absolute and relative circadian profiles of urinary aMT6s in those 11½-week-old male and female gerbils from the HF and LF groups which urinated during every 3-h interval, i.e., data were not averaged out. Data expressed as mean ± SEM, n = 5-11

7.1.2 Circadian Profiles of aMT6s in Gerbil Urine at 16 Weeks

Table 7.2 presents the mean urinary aMT6s levels excreted during 3-h intervals throughout 24-h by male and female gerbils aged 16 weeks from the HF and LF groups; LD: 12 12.

Table 7.2 Summary of circadian profiles of urinary aMT6s (ng/3-h and pg/g BW/3-h) in male and female gerbils aged 16 weeks from both groups; data expressed as mean ± SD, n = 12

	aMT6s ng/3 hours				**aMT6s pg/g BW/3 hours**			
	16 week high-F females	16 week high-F males	16 week low-F females	16 week low-F males	16 week high-F females	16 week high-F males	16 week low-F females	16 week low-F males
Time	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD
1600 - 1900	1.5 ± 1.0	2.1 ± 0.7	2.4 ± 1.0	3.2 ± 1.4	22 ± 13	27 ± 6	36 ± 15	37 ± 16
1900 - 2200	1.7 ± 1.4	2.7 ± 1.0	2.3 ± 1.4	2.8 ± 1.4	25 ± 15	35 ± 13	34 ± 22	33 ± 16
2200 - 0100	3.2 ± 2.4	4.1 ± 3.1	2.5 ± 1.4	3.2 ± 1.7	45 ± 34	51 ± 35	37 ± 24	38 ± 20
0100 - 0400	4.7 ± 3.8	6.0 ± 3.5	5.7 ± 2.8	7.2 ± 4.5	69 ± 51	76 ± 42	83 ± 40	83 ± 47
0400 - 0700	6.8 ± 4.2	8.3 ± 4.2	8.6 ± 4.5	8.9 ± 6.2	98 ± 56	106 ± 49	126 ± 66	101 ± 66
0700 - 1000	3.2 ± 2.4	3.8 ± 3.1	3.7 ± 2.4	4.4 ± 3.1	45 ± 29	48 ± 35	55 ± 37	52 ± 39
1000 - 1300	2.2 ± 1.4	2 3 ± 0.7	2.7 ± 1.4	2.1 ± 0.7	31 ± 13	30 ± 8	41 ± 20	25 ± 6
1300 - 1600	1.5 ± 0.3	2.2 ± 0.7	2.1 ± 0.7	2.3 ± 0.7	23 ± 5	28 ± 6	31 ± 10	27 ± 7
mean aMT6s ng/24 h	24.9 ± 11.6	31.7 ± 11.1	29.9 ± 8.2	34.1 ± 14.5				
body weight g	68 ± 9.9	78 ± 6.1	67 ± 5.5	85 ± 9.5				
mean aMT6s pg/g BW/24 h	356 ± 149	400 ± 112	444 ± 126	397 ± 148	355 ± 149	400 ± 112	444 ± 125	397 ± 149

Figure 7.7 illustrates that all 16-week-old gerbils exhibited well-defined circadian rhythms of aMT6s with peak levels occurring between 0400-0700 and low levels during the daytime, irrespective of sex or F-intake.

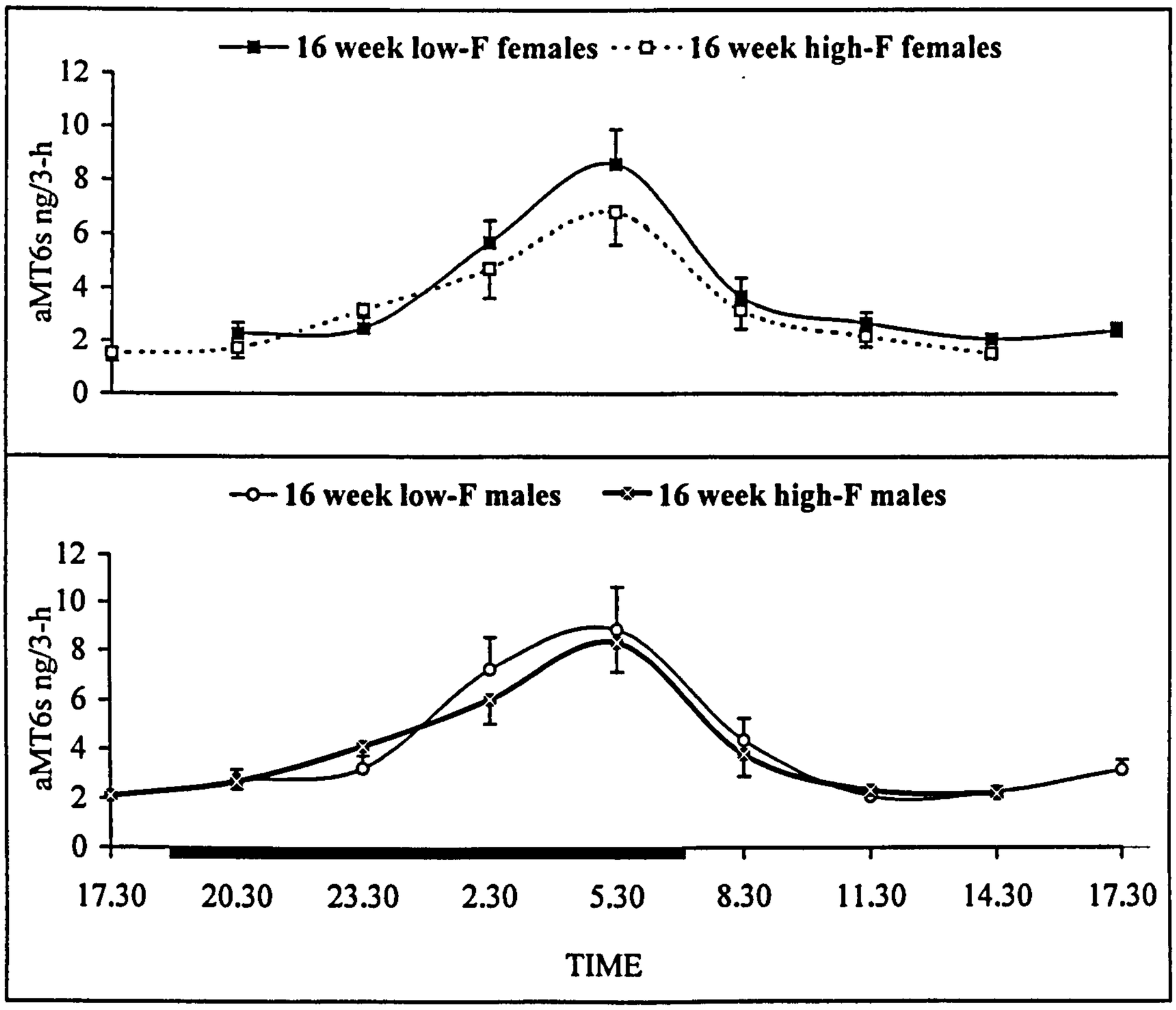

Fig. 7.7 Comparison of the circadian profiles of aMT6s (ng/3-h) in gerbils from the HF and LF groups at 16 weeks. LD: 12 12. Black bar indicates darkness; labels on the x-axis represent the mid-points of the 3-h intervals; data points represent mean ± SEM, n = 12.

Figure 7.8 shows that all 16-week-old gerbils produced similar relative circadian profiles of urinary aMT6s irrespective of sex or F-intake. They excreted similar amounts of aMT6s during the 12-h night period (2200 to 1000) with peak values of aMT6s between 0400 to 0700.

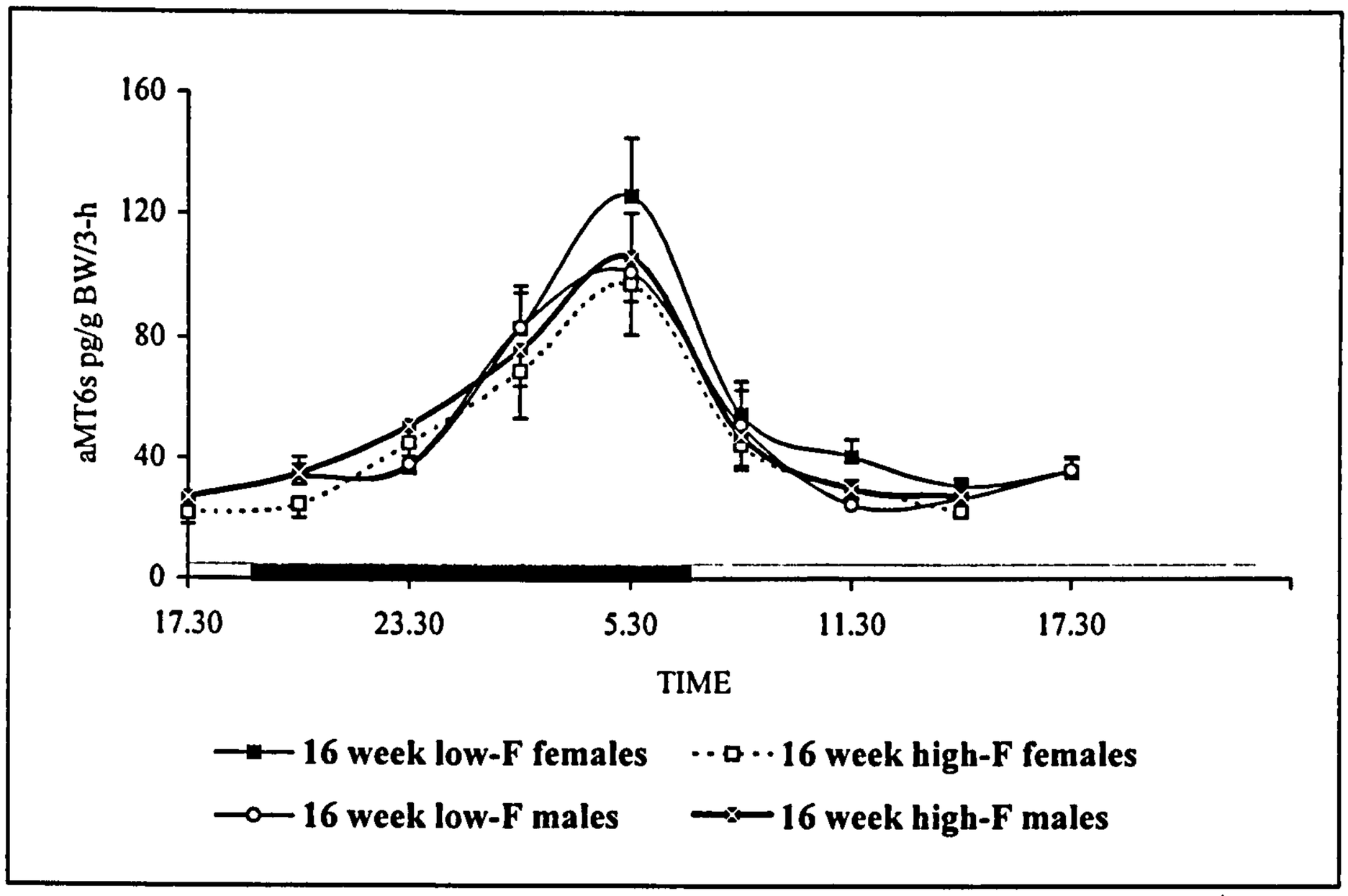

Fig. 7.8 Comparison of the relative circadian profiles of urinary aMT6s in male and female gerbils aged 16 weeks from the HF and LF groups. LD: 12 12. Black horizontal bar indicates period of darkness; data points represent mean ± SEM, n = 12.

7.1.3 Comparison of the Circadian Profiles of Urinary aMT6s in Gerbils at 11½ and 16 Weeks

Figure 7.9 shows that the 11½-week profiles of aMT6s in the LF group, (both males and females), were similar to those at 16 weeks. Indeed, the circadian profiles in LF males at 11½ and 16 weeks were indistinguishable. There appeared to be a phase shift in the relative profile in the LF females, i.e., the circadian profile at 16 weeks lagged behind the 11½-week-profile. At 11½ and 16 weeks, the LF males produced relative profiles which retained a good temporal pattern with each other although the AUC was smaller at 16 weeks than at 11½ weeks.

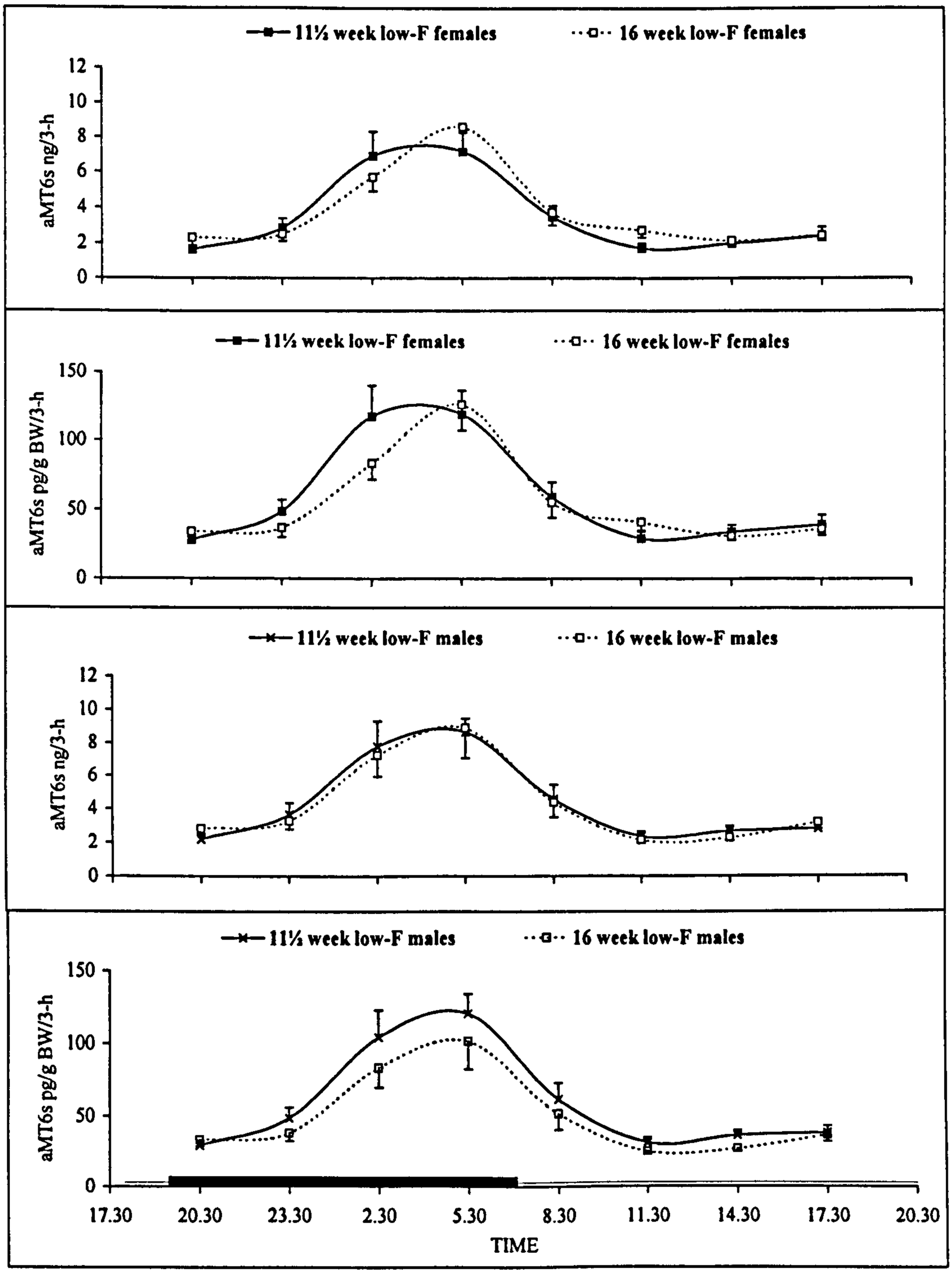

Fig. 7.9 Comparison of the absolute and relative circadian profiles of urinary aMT6s in male and female gerbils from the LF group at 11½ and 16 weeks. LD: 12 12. Black horizontal bar indicates period of darkness; data points represent mean ± SEM, n = 12.

Figure 7.10 compares the mean circadian profiles of aMT6s in eleven HF females at 11½ and 16 weeks. The peak values of aMT6s (ng/3-h and pg/g BW/3-h) were similar at 11½ and 16

weeks but they appeared later during the dark phase at 16 weeks (0400-0700) than at 11½ weeks (0100-0400).

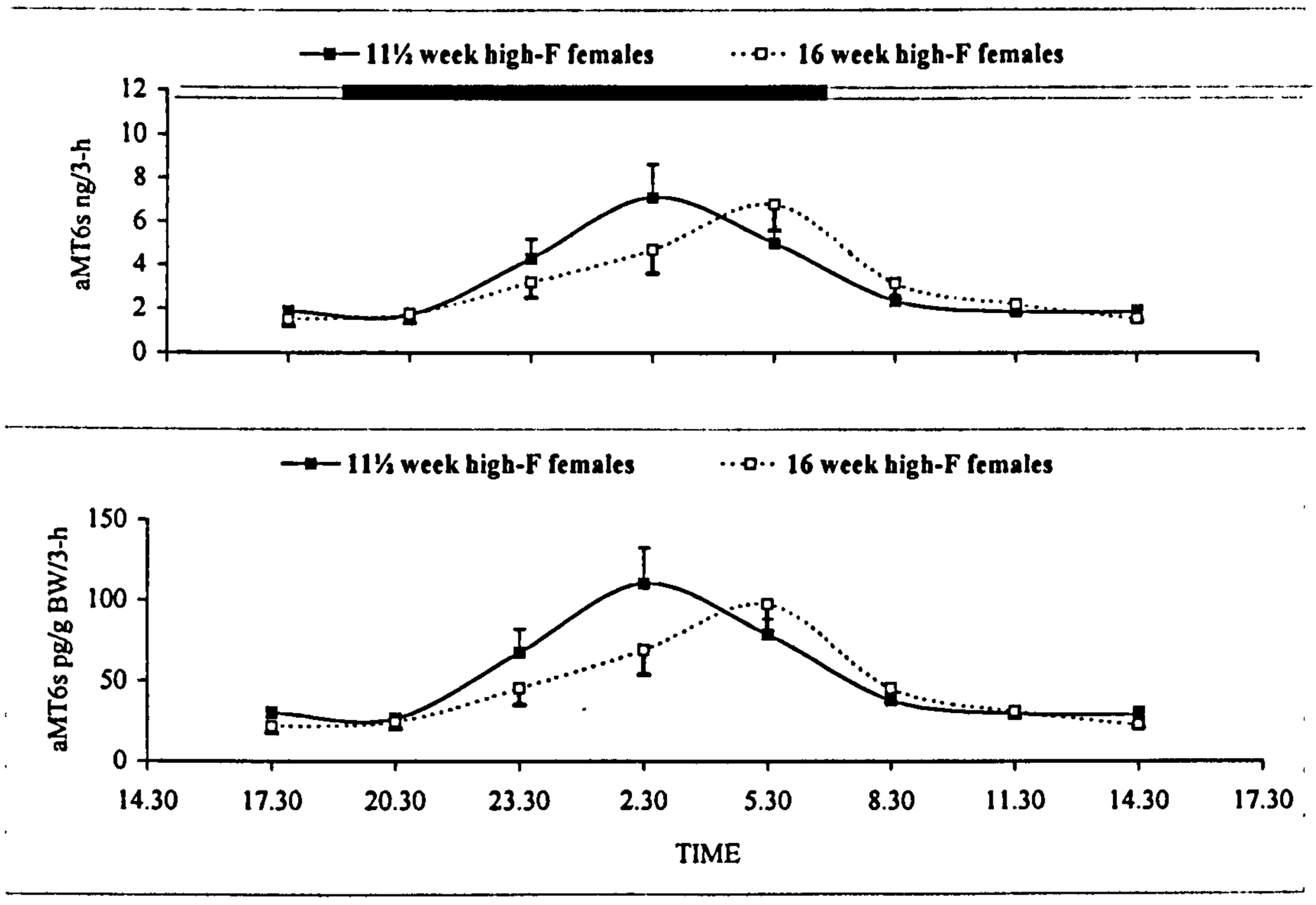

Fig. 7.10 Comparison of the mean absolute and relative circadian profiles of urinary aMT6s in HF female gerbils at 11½ and 16 weeks. LD: 12 12. Black horizontal bar indicates period of darkness; data points represent mean ± SEM, n = 11.

At 16 weeks, the HF females excreted daytime values of aMT6s during the first few hours of the night, i.e., the duration of their elevated values of aMT6s was shorter at 16 weeks than at 11½ weeks.

Figure 7.11 compares the circadian profiles of aMT6s in HF males at 11½ and 16 weeks. The aMT6s profile by HF males was clearly less robust at 16 weeks compared to 11½ weeks. Between 0400-0700, the 16-week-old HF males excreted significantly more aMT6s than at 11½ weeks: 8.3 ± 4.0 vs. 3.5 ± 1.5 ng/3-h,

respectively: $p < 0.001$; in relative terms, 106 ± 49 *vs.* 48 ± 21 pg/g BW/3-h, respectively: $p < 0.001$. There was a phase shift in peak nocturnal values of aMT6s. At 11½ weeks, the HF males excreted the highest values of aMT6s between 0100-0400; at 16 weeks, they excreted the highest aMT6s values between 0400-0700.

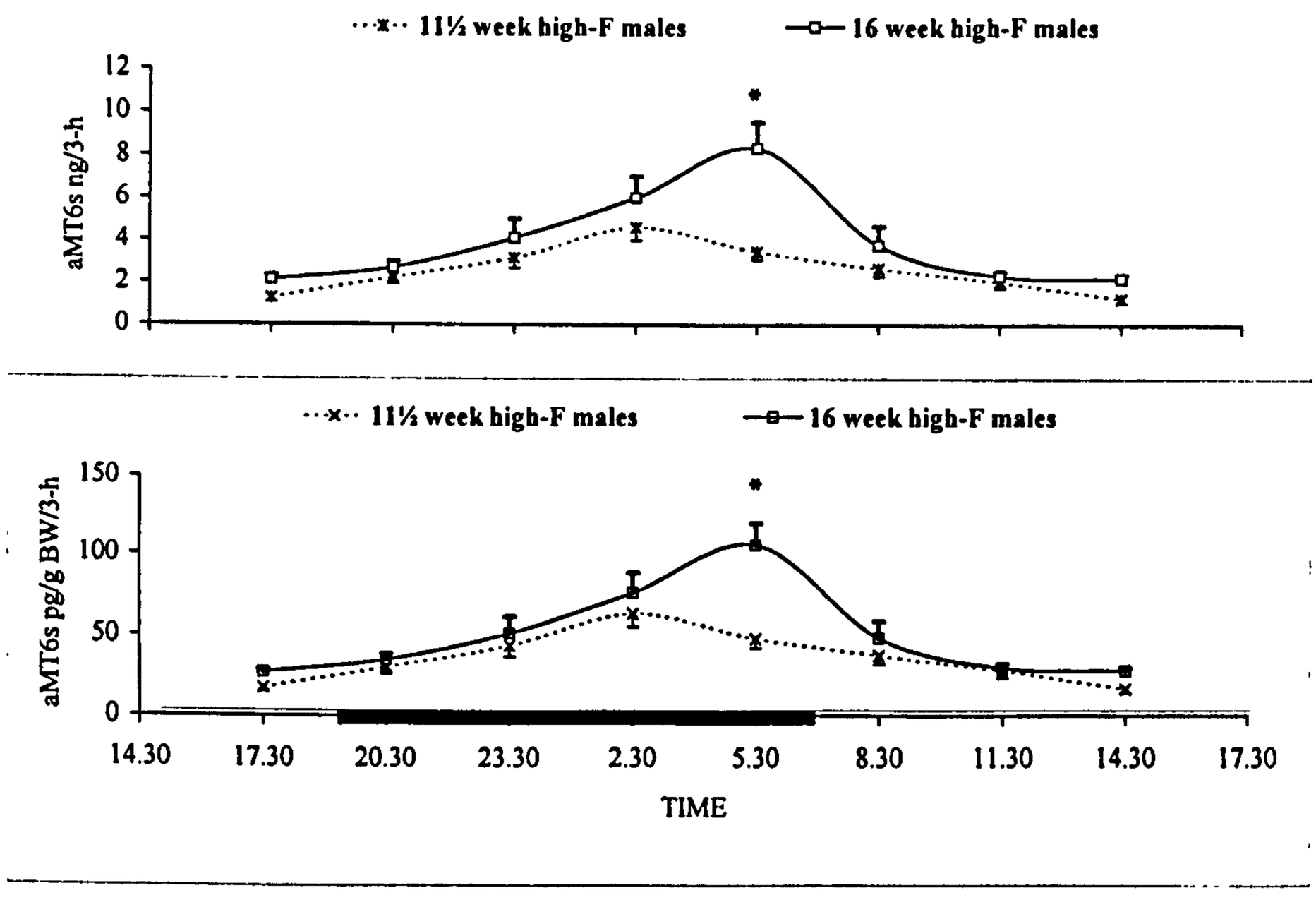

Fig. 7.11 Comparison of the mean absolute and relative circadian profiles of urinary aMT6s in HF male gerbils at 11½ and 16 weeks of age. LD: 12 12. Black horizontal bar indicates period of darkness; data points represent mean ± SEM, n = 12; *$p < 0.001$.

7.1.4 Intervals When the Gerbils Did Not Urinate

Table 7.3. presents the number of 3-h intervals when gerbils did not urinate. At 11½ weeks, the first 3-h interval was ignored because the gerbils often urinated prior to being placed in the metaboles. Therefore, the total number of 3-h intervals over 48-h was 15 per subject. When n = 12, total number of intervals per group = 180;

when n = 11, (HF females), total number of intervals per group = 165. At 16 weeks, the number of 3-h intervals over 24-h was 8 per subject. Therefore, when n = 12, total number of 3-h intervals = 96. (At 16 weeks, the first interval was not ignored because the gerbils were already acclimatised to the metaboles).

Table 7.3 Comparison of the number of 3-hourly intervals when the gerbils did not urinate

	11½ weeks			**16 weeks**		
	Number of gerbils			Number of gerbils		
Sample	void	non-void	total no	void	non-void	total no
low-F females	149	31	180	77	19	96
high-F females	148	17	165	71	25	96
	11½ weeks			**16 weeks**		
	Number of gerbils			Number of gerbils		
Sample	void	non-void	total no	void	non-void	total no
low-F males	156	24	180	81	15	96
high-F males	142	38	180	66	30	96

The Chi-squared test with Yates' correction was used to test whether F was associated with an increase in the number of intervals when the gerbils did not urinate. At 16 weeks, the HF males urinated significantly less frequently than the LF males: $p < 0.03$. At 11½ weeks, the LF females urinated less frequently than the HF females although this was not significant: $p < 0.06$.

7.2 Conclusions

At 11½ and 16 weeks, all gerbils exhibited pronounced circadian rhythms of urinary aMT6s which were characterized by high values during the night-time and low values during the daytime. At 11½ weeks, the HF males had a severely dampened rhythm. Compared

to the 11½-week-old LF males, they excreted significantly lower nocturnal values of aMT6s ($p < 0.05$) and 63% less aMT6s during the dark phase (2200-1000), i.e., 24.0 ± 8.7 *vs.* 15.2 ± 4.1 ng/12-h (± SD), respectively. (Figs. 7.1 and 7.2). After correction for body weight, the 11½-week-old HF males excreted significantly less aMT6s between 0100-0400 than the 11½-week-old HF females: $p < 0.05$. (Fig. 7.4). In addition, the circadian profile in the HF males was far less robust at 11½ weeks than at 16 weeks, i.e., half the amplitude, a shorter duration of elevated nocturnal values, and lower peak values ($p < 0.001$) which occurred between 0100-0400 at 11½ weeks and 0400-0700 at 16 weeks. (Fig. 7.11).

By 16 weeks of age, all the gerbils produced similar circadian profiles of aMT6s. (Figs. 7.7 and 7.8). This suggests that all young adult gerbils, even the HF males, have similar pineal outputs of MT. The inhibitory effects of F on pineal MT synthesis in the male gerbil ceased sometime after 11½ weeks and allowed the enzymic activity in the gerbil pineal to increase to normal values by 16 weeks of age. Obviously, the HF males would have progressively increasing plasma MT levels during this time.

In contrast, the LF males exhibited identical absolute profiles of urinary aMT6s at 11½ and at 16 weeks although the AUC of the relative profile was smaller at 16 weeks than at 11½ weeks. (Fig 7.9). This suggests that the LF male gerbil pineal secretes a constant output of MT with a uniform rhythm for a few weeks after sexual maturation. From 11½ to 16 weeks, the body weight of the LF males increased from 73 to 80 g which dilutes circulating MT. Therefore, the LF male gerbils had decreasing levels of plasma MT

following puberty. In direct contrast, the HF males had increasing plasma MT levels during this period.

At 11½ weeks, the LF males and LF females produced similar aMT6s profiles. The LF males had a higher absolute rate of aMT6s excretion than the LF females but this was not significant. In fact, after correction for body weight, the profiles by the LF males and females were virtually indistinguishable. (Fig. 7.3). The LF males and LF females excreted almost identical amounts of aMT6s between 2200-1000, i.e., 326 ± 105 *vs.* 340 ± 78 pg/g BW/12-h, respectively. There is no sex difference in pineal MT synthesis in gerbils at 11½ weeks.

However, at 11½ weeks, the HF females produced a similar relative profile of aMT6s as the LF males and LF females. (Fig. 7.5). They also excreted similar levels during the night-time (2200-1000) as the 11½-week-old LF males and LF females, i.e., 291 ± 122 *vs.* 326 ± 105 and 340 ± 78 pg/g BW/12-h, respectively. Female gerbils exhibited similar absolute and relative profiles of aMT6s at 11½ weeks and 16 weeks, irrespective of their F-intake. Female gerbils took longer to reach peak values of aMT6s at 16 weeks than at 11½ weeks. (Figs. 7.1, 7.2, 7.6, 7.7 and 7.8). These results are in agreement with results from Chapter 6, i.e., HF and LF females excreted similar total aMT6s at 11½ and 16 weeks. Fluoride does not affect the ability of the pineal to secrete MT after sexual maturation in the gerbil.

The use of 3-h excretion rates of urinary aMT6s to investigate the circadian rhythm of pineal MT synthesis depends upon a steady

rate of urine production by the subjects. Therefore, in those species which consistently excrete large quantities of urine, 3-h urine collections for the measurement of urinary aMT6s levels is a satisfactory method of monitoring pineal MT rhythms. Unfortunately, this criterion does not apply to gerbils. In this study, some gerbils did not urinate during every 3-h period (occasionally not during two 3-h intervals). Therefore, to compensate, the urinary aMT6s value excreted in the subsequent 3-h interval was averaged over six hours and very occasionally over nine hours.

Fig. 7.6 only includes gerbils which voided in every 3-h interval and so presents a clearer representation of the profiles of aMT6s. At 11½ weeks, the circadian profiles in the LF males, LF females and the HF females were similar and typically showed a five-fold amplitude. The HF males produced a aMT6s profile with a two-fold amplitude. Unfortunately, there was not enough time to determine the aMT6s profiles by gerbils at 7 and 9 weeks. However, at 7, 9 and 11½ weeks, the HF males excreted significantly less total aMT6s than the LF males. In addition, in terms of body weight, the HF males excreted similar total aMT6s at 7, 9 and 11½ weeks, i.e., 308, 320 and 299 pg/g BW/24-h, respectively. (Table 6.1). Therefore, the HF males presumably had similar aMT6s profiles at 7, 9 and 11½ weeks. If so, the 7- and 9-week-old HF males exhibit aMT6s profiles with a two-fold amplitude. This is consistent with the two-fold amplitude in pineal MT content in 8-week-old male gerbils reported in a previous study (Reiter *et al*, 1980).

Stock rat food made with 'natural ingredients' can contain high levels of F because bone meal is added to provide calcium: e.g., Wayne rodent blox, Purina laboratory chow and Teklad 6% rat/mouse diet contains 27.5, 11.6 and 45.2 mg F/kg respectively. Furthermore, the bioavailability of F in these rodent foods is 45-50%. (Whitford, 1991). It is possible that the 8-week-old male gerbils used in Reiter's study (1980) were fed a stock rodent food with a high F-content and tap water at 1 mg F/L. The increased plasma levels of F (which would have been higher than the LF male gerbils used in the current study) may have inhibited pineal MT synthesis and resulted in the observed two-fold amplitude in pineal MT content. Therefore, Reiter's results would agree with the estimated two-fold amplitude of the aMT6s profile by age-matched HF males in the current study.

This is a convenient time to re-evaluate the data from the additional group of 9-week-old LF males which were not included in the statistical analyses. (Chapter 6). They excreted 15.3 ± 2.0 ng aMT6s/24-h: a significantly diminished rate of aMT6s excretion ($p < 0.001$) than the 9-week-old LF males from the longitudinal study (27.9 ± 7.7 ng/24-h). The additional 9-week-old LF males may have inadvertently received water intended for the HF group, (50 mg F/L), instead of distilled water, during the 48-h in the metaboles. The F-intake from the water coupled with F from the regular rodent food (LAB animal diet No. 1, from Lillico, UK contains 18.67 mg F/kg, see footnote § on p. 157) would increase the plasma F-levels. If this were so, then it suggests that transient high plasma F-levels may inhibit pineal MT output as effectively as chronically-increased plasma F-levels in prepubescent gerbils.

One reason why the human pubertal studies on pineal MT output have produced conflicting results may be that the investigators did not consider the F-intake to the children, e.g., whether or not the study was undertaken in a fluoridated area. In addition, it may be advisable to determine the F-contents of stock rodent feeds given to animals used in pineal research.

Fluoride may affect the clearance rate of aMT6s by the kidneys or the rate of MT metabolism in the liver with subsequent alterations in the levels of urinary aMT6s. A recent comprehensive study to determine whether aging increases the biological impact of F included the monitoring of the levels of blood indicators of physiological 'wellness' from 4 groups of rats fed 0, 5, 15 or 50 mg F/L in their drinking water for 18 months (Dunipace *et al*, 1995). Any alterations in values of serum glutamate oxaloacetate transaminase, creatinine, urea nitrogen, glucose, bilirubin, would indicate detrimental effects of F on kidney or liver function. The investigators found no adverse physiological or genotoxic effects from F. None of the monitored markers of tissue integrity and function was altered by F. The urine urea and creatinine data for the rats on 50 mg F/L were significantly greater ($p < 0.05$) than the other groups. If F caused an impairment of kidney function, these indicators of kidney function would have decreased rather than increased. In addition, the histopathological changes in liver and kidney specimens at 18 months occurred across all groups.

In the current study, the 16-week-old HF males urinated significantly less frequently than similarly aged LF males ($p < 0.03$). (Table 7.3). These results are difficult to interpret. If F

affected kidney function why was this limited to 16-week-old males? If F had detrimental effects on liver or kidney function, then the 16-week-old HF males would not have had a normal aMT6s profiles. Conversely, when the profiles of aMT6s in the HF and LF males were significantly different (at 11½ weeks), there was no significant difference in the frequency of urination between the LF and HF males. In addition, at 11½ weeks, the HF females urinated more frequently than the LF females ($p < 0.06$). This evidence diminishes the possibility that the differences in the rates of aMT6s excretion between the groups is due to the effects of F on kidney or liver function although this area needs further investigation. It may be that the 16-week-old HF males simply limited their water consumption. They may have had an aversion to the water supplied during the 48-h in the metaboles (50 mg F/L) because they were used to drinking distilled water at other times. Indeed, they are physiologically capable of surviving for more than 45 days without water (Boice and Witter, 1970).

The amplitude of the night-time peak in aMT6s values varied from one individual to the next. At 11½ weeks, gerbils excreted a wide range of peak nocturnal values, i.e., 1.9-19.6 ng aMT6s/3-h (data taken from gerbils which urinated in every 3-h interval but excluding the HF males). Although some gerbils had a much higher nocturnal rate of aMT6s excretion than others, they all excreted the same rate of urinary aMT6s (about 2-3 ng/3-h) during the daytime, irrespective of gender, treatment or age. Within an individual, the rhythm was stable and reproducible over two consecutive days. There was a good correspondence between the mean profiles,

nocturnal values and total aMT6s excreted in day 1 and day 2 across the groups. (Table 7.1).

In conclusion, the 11½-week-old HF males exhibited a circadian profile of urinary aMT6s which was significantly diminished from: a) their own at 16 weeks; b) the 11½-week-old HF females; c) the 11½-week-old LF group. There were changes in the temporal pattern, diminished duration and reduced amplitude of the rhythm. The results suggest that F inhibits the synthesis of pineal MT during the dark phase in HF males at 11½ weeks. Between 11½ and 16 weeks, the MT message that the pineal sends to every cell in the HF males is unusual: increasing plasma MT levels between sexual maturity and young adulthood. At 11½ and 16 weeks, the HF females and LF females exhibited similar profiles. This suggests that the inhibitory effects of F on pineal synthesis of MT cease after the HF females are sexually mature.

§All gerbils were fed regular rodent food (LAB animal diet, No 1, obtained from Lillico, UK) during the 48 hours in the metaboles because their specially-prepared food was in the form of small pellets which could fall through the rack. LAB animal diet, No 1 contained 18.67 mg F/kg (kindly analysed by Dr J Toumba, The Leeds Dental Institute).

CHAPTER 8 - Effects of Fluoride on the Physiological Signs of Puberty in Gerbils

8.1 Results of the Areas of the Ventral Glands

Table 8.1 Ventral gland formation in female gerbils aged 11½ weeks from the LF and HF groups.

Group	Number of gerbils	
	Developed ventral glands	Undeveloped ventral glands
High-F females at 11½ weeks	7	3
Low-F females at 11½ weeks	4	21

At 11½ weeks, 21 (84%) of 25 LF females had indistinct, un-differentiated ventral glands which were not measurable and the mean area of the four differentiated glands was 0.4 cm^2. At 11½ weeks, three (30%) of ten HF females had indistinct ventral glands and the mean area of the seven differentiated glands was 0.4 cm^2. There was a highly significant difference in the occurrence of a developed, differentiated gland at 11½ weeks: using Fisher's Exact Test, two-tailed, $p < 0.004$. At 28 weeks, all HF and LF females had well-differentiated ventral glands: areas were 0.59 ± 0.08 and 0.58 ± 0.12 cm^2 respectively, (mean ± SD). All males from the HF and LF groups had well-differentiated ventral glands: at 9 weeks, the areas were 1.03 ± 0.2 cm^2 and 0.95 ± 0.18 cm^2 respectively; at 28 weeks, the areas were 1.28 ± 0.30 and 1.19 ± 0.28 cm^2 respectively. See Table E.1 in Appendix.

8.2 Results of Body Weights

Table 8.2 Body weights (g) of gerbils from 7 to 28 weeks

	Age (weeks)				
Group	7	9	11½	16	28
high-F females	50.8 ± 5.6	55.2 ± 4.0	62.7 ± 7.2	67.9 ± 9.1	75.6 ± 6.2
low-F females	44.8 ± 3.0	54.0 ± 3.4	59.3 ± 4.7	66.1 ± 6.1	70.2 ± 4.3
P-value, student's t-test	< 0.004	< 0.5	< 0.04	< 0.5	< 0.01
Power	0.85	-	0.55	-	0.66
95% confidence intervals	2.2 - 9.8	-	0.1 - 6.7	-	1.2 - 9.6
N (in order HF, LF)	12, 12	12, 12	24, 30	16, 18	13, 14
high-F males	54.0 ± 5.8	61.4 ± 4.7	69.7 ± 5.4	76.8 ± 6.4	91.1 ± 6.9
low-F males	54.1 ± 3.0	64.3 ± 5.1	73.2 ± 6.9	80.4 ± 9.0	95.2 ± 18.1
P-value	< 1.0	< 0.07	< 0.1	< 0.2	< 0.6
N (in order HF, LF)	12, 12	20, 19	21, 12	20, 21	8, 9

The LF females weighed less than the HF females throughout: significantly so at 7, 11½ and 28 weeks of age. The HF males weighed less than the LF males throughout but not significantly so. The males were heavier than the females at each age except at 7-weeks, when the HF females, HF males and LF males had similar body weights: $df = 2, 33$; $F = 1.76$; $p < 0.2$.

Fig. 8.1 presents the rates of growth of gerbils from 7 to 28 weeks of age. The male and female gerbils had an increase in the rate of growth from 7 to 12 weeks. From 12 to 28 weeks the rate of growth tends to decline irrespective of sex. At 28 weeks, the LF males were about 24% heavier than the LF females; whereas the HF males were about 14% heavier than the HF females.

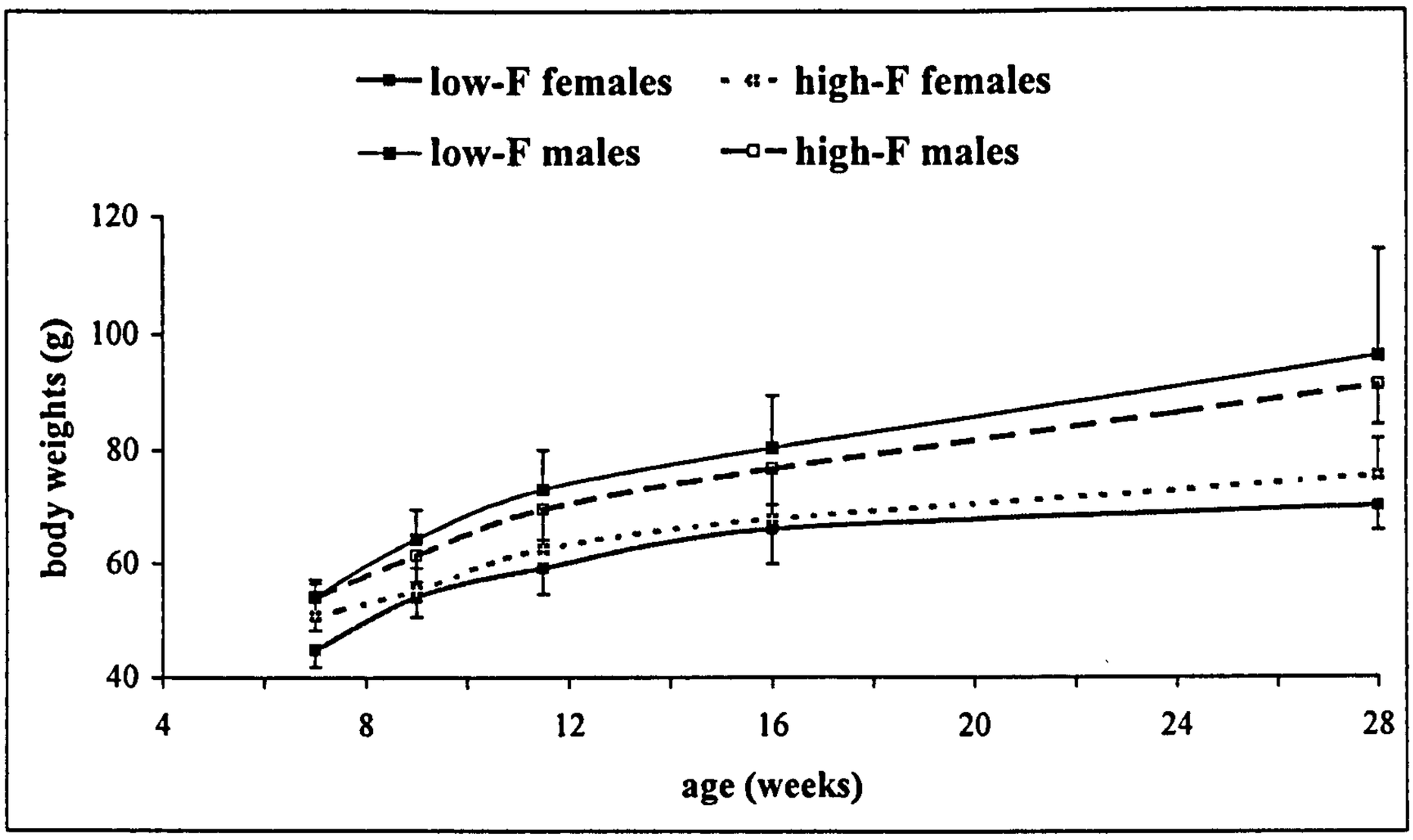

Fig. 8.1 Body weights (g) of male and female gerbils from the HF and LF groups from 7 to 28 weeks of age. Data expressed as mean ± SEM, n = 8 - 30.

8.3 Results of the Age at Vaginal-Opening

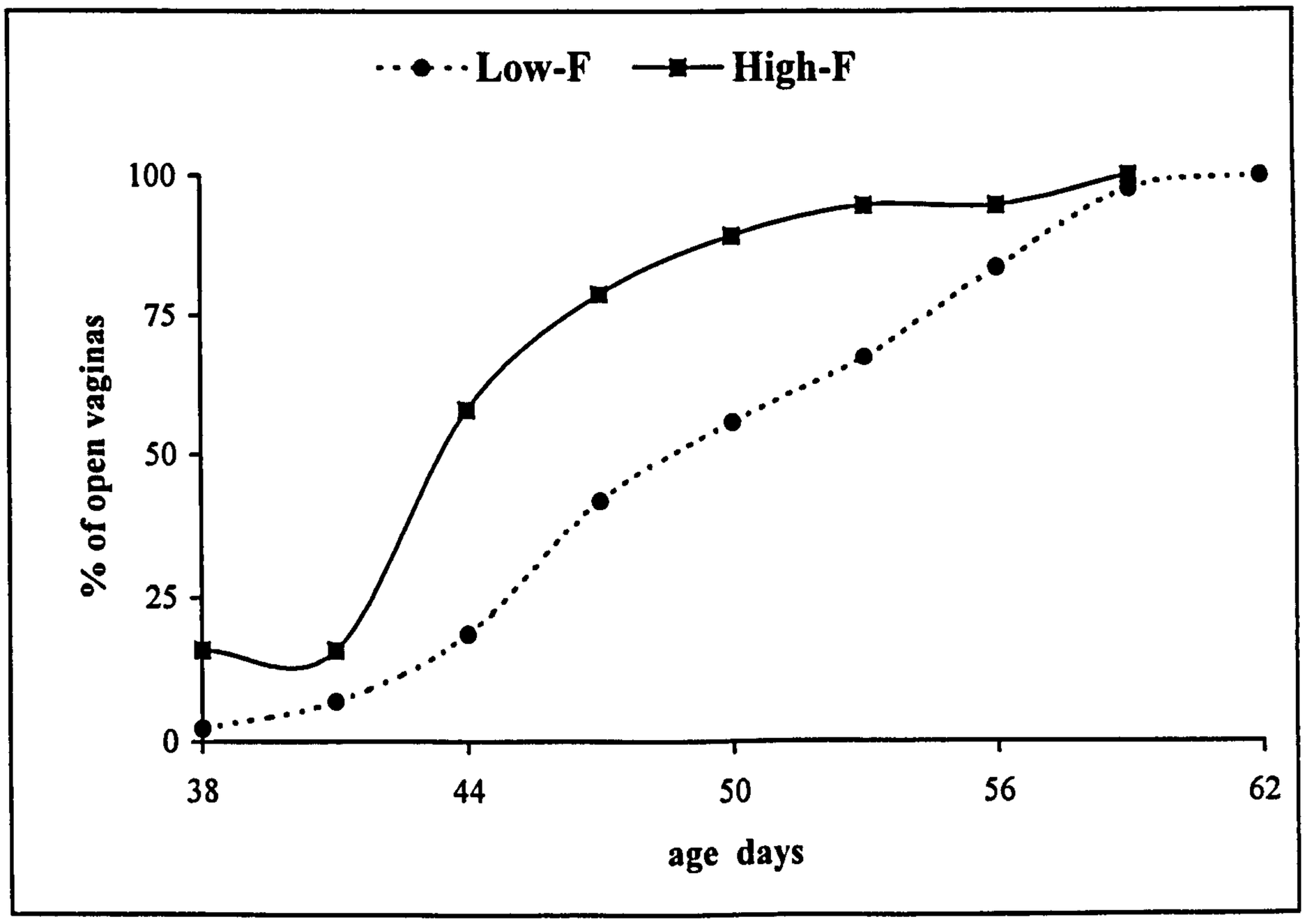

Fig. 8.2 Cumulative percentage of age at vaginal opening in the high-F females, (n = 19), and low-F females, (n = 43).

Figure 8.2 shows the cumulative distribution of age at vaginal opening in females from the HF and LF groups. Vaginal opening occurred significantly earlier in the HF females than the LF females. Table 8.3 compares the number of female gerbils from the HF and LF groups showing vaginal opening at 7 weeks: using Chi-square test, applying Yates' correction, $p < 0.025$.

Table 8.3 Comparison of the number of females showing vaginal introitus at 7 weeks.

Sample group	Number of gerbils		
	open vaginas	closed vaginas	N
High-F females at 46-48 days	15	4	19
Low-F females at 46-48 days	18	25	43

8.4 Results of Testes Weights

Table 8.4 presents the combined testes weights of the HF and LF males at 9, 16 and 28 weeks of age. There were no significant differences between the combined testes weights of the HF and LF males at 9 and 28 weeks of age. At 16 weeks, the mean combined testes weight of the HF males was significantly lower than that of the LF males: $df = 1, 22$, $F = 12.925$, $p < 0.002$. Power of a t-test (with a 5% risk of a Type 1 error) to detect the observed mean difference of 223 mg was 0.98. The 95% confidence interval for the mean change in testes weights was 95 to 350 mg.

See Table E.4 in Appendix.

Table 8.4 Combined testes weights (g) of male gerbils at 9, 16 and 28 weeks. Data expressed as mean ± SD; N in parentheses.

	9 weeks	16 weeks	28 weeks
high-F males	0.94 ± 0.14 [11]	1.10 ± 0.11 [12]	1.24 ± 0.06 [5]
low-F males	0.94 ± 0.08 [7]	1.32 ± 0.18 [12]	1.15 ± 0.12 [9]

8.5 Conclusions

This section of the work must be regarded as a preliminary, pilot study as there were too few animals to make any firm conclusions. However, the results suggest that the HF females reached puberty earlier than the LF females. At 7 weeks of age, the HF females weighed significantly more than the LF females ($p < 0.004$); in fact they were as heavy as the HF and LF males. If the rate of attainment of body weight is relevant to puberty in gerbils, it would appear that the HF females have entered their pubertal growth spurt and attained a critical weight earlier then the LF females. It is difficult to explain why the HF female gerbils were significantly heavier ($p < 0.01$) than the LF females at 28 weeks. It is well known that hyperglycaemia is associated with acute fluoride toxicity. In humans, the pineal undoubtedly plays a part in carbohydrate metabolism and there is evidence that pineal dysfunction is involved in the development of *diabetes mellitus*.

As figure 8.2 suggests and the statistical test confirms, the HF females exhibited vaginal opening at an earlier age than the LF females. However, if vaginal opening in gerbils is unrelated to the

age at first successful reproduction, (Clark and Galef, 1985), it may not be a satisfactory index of rate of sexual development in the gerbil. The ventral glands of the HF females also developed significantly earlier than the LF females ($p < 0.004$): the ventral glands in the LF females were still in a primordial state at 11½ weeks. The accelerated growth of the ventral glands in the HF females suggests they are sexually mature earlier than the LF females. It has been suggested that the appearance of the gland in gerbils gives a good indication of functional ovaries and uteri and is correlated to sexual maturation (Swanson and Lockley, 1978). The gland develops earlier in the male gerbil so any difference in the size or appearance of the gland between the HF and LF males was missed in this study. The ventral glands in the males should have been measured at 5-6 weeks. At 16 weeks, the mean testes weight in the HF males was significantly less than the LF males $p < 0.002$). This could be due to the different circulating MT levels during the month from sexual maturity to adulthood: the LF males had progressively decreasing plasma MT levels whereas the HF males increasing plasma MT levels. This could conceivably influence pineal-gonadal interaction. It raises the question whether there is a drop in spermatogenic capacity in the adult HF males.

In conclusion, assuming the validity of the hypothesis that high nocturnal plasma MT levels during early development act as a break on the hypothalamic-pituitary-gonadal axis, then the HF gerbils would enter puberty earlier than the controls. They have lower circulating MT levels. It sounds biologically plausible. However, more work needs to be done in this area before any firm conclusions can be made.

CHAPTER 9 - Fluoride Levels in Gerbil Bone

9.1 Results

Table 9.1 presents the mean F-content (mg/kg) in gerbil bone at 10 (11), 16 and 28 weeks.

Table 9.1 Summary of the mean (± SD) F-content of gerbil bone ash (mg/kg) at 10 (11), 16 and 28 weeks; n = 5 - 16.

Females	11 weeks	16 weeks	28 weeks
Low-F females	365 ± 79	579 ± 85	727 ± 145
High-F females	1481 ± 197	2692 ± 538	2783 ± 274
High-F females HFAW	1310 ± 33		
Males	**10 weeks**	**16 weeks**	**28 weeks**
Low-F males	443 ± 55	579 ± 85	586 ± 61
High-F males	1804 ± 170	2461 ± 198	2781 ± 95
High-F males HFAW	1286 ± 106		

N.B. HFAW represents a group of gerbils which received a high-F diet after weaning at 24 days.

The highest [F] (2800 mg/kg) were found in the bone ash from the 28 week-old high-F gerbils. There was no sex difference. The mean [F] in male and female bone from the HF group at 16 weeks were 2460 ± 198 and 2690 ± 538 mg/kg respectively. There was no significant difference between the sexes at 16 weeks ($p = < 0.3$).

Bone ash from 10-week-old males from the HF group contained 1800 ± 170 mg F/kg compared to 1290 ± 100 mg F/kg in bone ash from a group of 10-week-old male gerbils which had received the high-F food only after weaning at 24 days. Bone ash from 11-week-old females from the HF group contained 1480 ± 200 mg F/kg compared to 1310 ± 30 mg F/kg in bone ash from a group of 11-week-old female gerbils which had received the high-F food only after weaning at 24 days. The 10-week-old males retained significantly more F than the 11-week-old females ($p < 0.002$) despite the age difference.

The mean [F] in bone ash from the LF group was much lower at all ages than those from the HF gerbils. Females: mean [F] in bone was 360 ± 80 mg/kg at 11 weeks increasing to 730 ± 150 mg /kg at 28 weeks. Males: mean [F] in bone was 440 ± 50 mg/kg at 10 weeks increasing to 590 ± 60 mg /kg at 28 weeks.

9.2 Conclusions

At each age, the mean [F] in bone ash from the LF group was about a third of those in the HF group. The [F] of gerbil bone was highly consistent within the groups in contrast to the [F] of human bone reported in Chapter 4 where there was considerable inter-individual variation. This was partly due to analysing trabecular bone (parietal) every time. In addition, the gerbils were genetically similar, had been exposed to identical environmental conditions; and more significantly their F-intake had been controlled.

The [F] of bone ash from the LF group was higher than I had anticipated. This could be due to i) the pups might have started gnawing and presumably ingesting stock rodent food (18.67 mg F/kg) intended for their parents at 18 days (age when eyes opening occurs in gerbils) until weaning at 24 days; ii) they received the stock rodent food (18.67 mg F/kg) during the 48-h in the metaboles; iii) specially prepared food was not semi-purified but made with natural ingredients without added bone ash (7 mg F/kg).

The [F] of the bone ash from the HF group was well within the range reported in previous investigations on [F] of bone ash in animal studies using various F-doses. See table 1.1. The lower [F] in bone ash from gerbils which had received high-F diet after weaning at 24 days compared to [F] in bone which received F from day 1 indicates that F is rapidly taken up by the growing skeleton neonatally. At 28 weeks, the LF females had higher bone [F] than the LF males. It may be due to the greater skeletal availability for F-deposition in the males because they were still growing: from 16-28 weeks, the body weight of the LF males increased by 15 g whereas the LF females increased by 4 g.

In conclusion, the only variable between the two groups was their F-intake.

Chapter 10 - Discussion

After half a century of the prophylactic use of fluorides in dentistry, we now know that fluoride readily accumulates in the human pineal gland. In fact, the aged pineal contains more fluoride than any other normal soft tissue. The concentration of fluoride in the pineal was significantly higher ($p < 0.001$) than in corresponding muscle, i.e., 296 ± 257 *vs.* 0.5 ± 0.4 mg/kg (wet weight) respectively. The low fluoride content found in muscle in the current study was in agreement with the low fluoride content in soft tissues - less than 1 mg F/kg (WHO, 1984). This indicates that the method used in the present study had been properly executed; that fluoride in the pineal gland was endogenous and had not been introduced to the cadavers since the time of death, e.g., via the preserving formalin fluid. However, the pineal gland is unique in that it can be classified as a soft or as a mineralizing tissue. In terms of mineralized tissue, the mean fluoride concentration in the pineal calcification was equivalent to that in severely fluorosed bone and more than four times higher than in corresponding bone ash, i.e., 8,900 ± 7,700 *vs.* 2,040 ± 1,100 mg/kg, respectively. The calcification in two of the 11 pineals analysed in this study contained extremely high levels of fluoride: 21,800 and 20,500 mg/kg.

There is increasing interest in the determination of essential and toxic elements in neurological tissues. Fluoride metabolism in CNS has not been systematically studied. It is generally agreed that the CNS is impervious to the effects of fluoride by virtue of the blood-brain barrier

(Whitford *et al*, 1979). The human pineal is outside the blood-brain barrier. The significance of this is not clear but it may be that the pineal needs to 'sample' the circulating blood. The results from this study are important because the pineal gland is obviously a hitherto unrealized target for chronic fluoride-toxicity.

The pineal fluoride content varied considerably between subjects (14-875 mg/kg) although it was directly correlated to pineal calcium content: $r = 0.73$, $p < 0.02$. Large amounts of calcium have been demonstrated in the pineals from young children. Indeed, the prevalence of pineal calcification in young children is higher than one may have been led to believe from radiological evidence alone (Tapp and Huxley, 1971; Reyes, 1982). In addition to its high calcium content, the pineal contains intracellular colloids, a high magnesium content (Krstic, 1976; Michotte *et al*, 1977; Allen *et al*, 1981); and a very profuse blood supply. These are all factors encouraging the acquisition of fluoride by soft tissues (WHO, 1970). High levels of magnesium, manganese, zinc and copper have been demonstrated in pineals which appear 'uncalcified' (Michotte *et al*, 1977). Therefore, it is likely that the child's pineal also accumulates fluoride although this needs verification. The deposition of fluoride within the child's pineal must be a recent phenomenon. The plasma-fluoride levels in young children are normally very low and what little there is is rapidly sequestered by the growing skeleton. The extensive use of fluorides in dentistry has caused an unprecedented increase in plasma-fluoride levels in infants and young children.

Any adverse physiological effects of fluoride depend upon the concentration at various tissue sites. Can pinealocytes function normally in close proximity to high concentrations of fluoride? One would predict

that a high local fluoride concentration would affect pinealocyte function in an analogous way that a high local fluoride concentration affects: i) bone cells, since histological changes have been observed in bone with 2,000 mg F/kg (Baud *et al*, 1978); ii) ameloblasts, since dental fluorosis develops following fluoride concentrations of 0.2 mg F/kg in the developing enamel organ (Bawden *et al*, 1992). The consequences are disturbances in the functions of bone and enamel, i.e., changes in structure (poorly mineralized bone and enamel). If the pineal accumulates fluoride at an earlier age than in previous decades, one would anticipate that a high local concentration of fluoride within the pineal would affect the functions of the pineal, i.e., the synthesis of hormonal products, specifically melatonin. The highest levels of pineal melatonin are produced during early childhood.

The controlled animal study carried out in this study produced compelling evidence that fluoride inhibits pineal melatonin output during pubertal development in the gerbil. The LF males and LF females excreted similar amounts of the melatonin metabolite, aMT6s, in urine from prepubescence (7 weeks), throughout puberty to young adulthood (16 weeks). For example, at 7 weeks, the LF males and LF females excreted 30.7 ± 7.9 and 26.8 ± 6.8 ng aMT6s/24-h, respectively; at 16 weeks, 31.6 ± 10.9 and 29.8 ± 8.2 ng aMT6s/24-h. There was no sex difference. These results agree with previous reports that the rates of urinary aMT6s excretion remain constant during human puberty with no sex difference (Young *et al*, 1988; Bojkowski and Arendt, 1990; Tetsuo *et al*, 1982).

When the data were corrected for body weight, the LF group excreted progressively less urinary aMT6s from 7 to 16 weeks ($p < 0.01$). The LF

males and LF females excreted significantly more aMT6s at 7 weeks: 569 ± 148 and 602 ± 168 pg/g BW/24-h than at 16 weeks: 397 ± 148 and 443 ± 126 pg/g BW/24-h, respectively. This unique pattern of urinary aMT6s excretion has also been demonstrated in human pubertal studies (Young *et al*, 1988; Rager *et al*, 1989; Bojkowski and Arendt, 1990). The LF males and LF females excreted similar total aMT6s from 7 to 16 weeks and their circadian profiles of urinary aMT6s were strikingly similar at 11½ and 16 weeks. This is in agreement with previous human studies which found no sex difference between the relative rates of aMT6s excretion during puberty.

The results of urinary aMT6s excreted by the LF gerbils during pubertal development are 'classical' in the sense that they are similar to those reported in several human pubertal studies. Therefore, the LF group represent 'normal' gerbils with respect to urinary aMT6s levels excreted during sexual maturation. That the results from the LF group were foreseen indicates that the experiment had been properly executed. Therefore, this project has produced useful baseline data on the rates of urinary aMT6s excretion by the gerbil which can be used in future investigations using measurements of urinary aMT6s as an alternative to pineal melatonin measurements. However, the exactitude of the results of the LF group accentuates the divergent results from the HF group.

At 7 weeks, the prepubescent HF males excreted almost half as much urinary aMT6s as the LF males: 16.4 ± 4.2 *vs.* 30.7 ± 7.9 ng/24-h: $p < 1.5E-05$; in relative terms, 308 ± 76 *vs.* 569 ± 148 pg/g BW/24-h, respectively: $p < 0.00002$. The HF males continued to excrete significantly less aMT6s than the LF males throughout puberty: at 9 weeks, 19.6 ± 4.7 *vs.* 27.9 ± 7.7 ng/24-h ($p < 0.004$); in relative terms,

320 ± 75 *vs.* 425 ± 113 pg/g BW/24-h, respectively ($p < 0.01$); at 11½ weeks, 21.9 ± 5.7 *vs.* 33.0 ± 9.8 ng/24-h ($p < 0.003$); in relative terms, 299 ± 74 *vs.* 449 ± 111 pg/g BW/24-h, respectively, ($p < 0.001$). By 16 weeks, the HF males excreted normal levels of aMT6s. Indeed, young adult gerbils excreted similar total aMT6s and exhibited similar circadian profiles of aMT6s, irrespective of gender or treatment.

At 7 weeks, the HF females also excreted significantly less aMT6s than the LF females, 18.1 ± 5.5 *vs.* 26.8 ± 6.8 ng/24-h, ($p < 0.002$); in relative terms, 359 ± 109 *vs.* 602 ± 168 pg/g BW/24-h, respectively, ($p < 0.0004$). Thereafter, the level of statistical significance between the rate of aMT6s excretion by the HF females and LF females progressively declined: at 9 weeks, $p < 0.02$; and at 11½ and 16 weeks, the HF females and LF females excreted similar total aMT6s with similar circadian profiles.

The HF group not only excreted significantly less urinary aMT6s than the LF group but the patterns of excretion were different from the LF group. After correction for body weight, the HF group had a uniform, constant rate of aMT6s excretion during sexual maturation: unlike the LF group which excreted progressively less aMT6s during puberty. At 7 weeks, the HF group (unlike the LF group) did not excrete their highest relative levels of urinary aMT6s. At 11½ weeks, the HF males produced a dampened circadian profile of urinary aMT6s with a diminished amplitude of nocturnal peak values, reduced duration of nocturnal elevated values and a shift in the temporal pattern. These changes would not be distinguishable from those observed following photoperiod manipulation. At 11½ weeks, the HF males excreted significantly less urinary aMT6s than the HF females: 21.9 ± 5.7 *vs.* 26.1 ± 9.5 ng/24-h, (p

< 0.05); in relative terms, 299 ± 74 *vs.* 407 ± 134 pg/g BW/24-h, respectively, ($p < 0.02$).

The project also demonstrated that urinary aMT6s levels reflect pineal melatonin output in the gerbil. By inference, from prepubescence to young adulthood, the gerbil pineal (male and female) normally secretes a constant output of melatonin although, after correction for body weight, there is a significant progressive decline in melatonin output with age. Fluoride inhibited the pineal synthesis of melatonin in prepubescent male and female gerbils. The inhibitory effects of fluoride on pineal melatonin output lasted longer in males than females. A 'normal' pineal melatonin output was produced by the HF females at 11½ weeks; by the HF males at 16 weeks.

Female gerbils reach functional sexual maturity earlier than male gerbils. Female gerbils can give birth as early as 72 days (Cheal, 1983) whereas male gerbils can be 130 to 140-days-old before they sire their first litters (Norris and Adams, 1972). The results suggest that fluoride inhibited pineal melatonin synthesis up until the time of sexual maturation in the gerbil. Fluoride did not inhibit pineal melatonin synthesis in female gerbils once they were sexually mature (at 11½ weeks) and allowed the pineal melatonin output to reach 'normal' values. Fluoride continued to inhibit pineal melatonin synthesis in male gerbils at 11½ weeks because male gerbils take longer to reach sexual maturity. Their pineal melatonin output only reached 'normal' values at 16 weeks of age.

The most plausible hypothesis for the observed significant decrease in the rate of urinary aMT6s excretion by the HF group is that fluoride

affects the pineal's ability to synthesize melatonin during pubertal development in the gerbil. Fluoride may affect the enzymatic conversion of tryptophan to melatonin. Although melatonin was the hormone investigated in this project, fluoride may also affect the synthesis of melatonin precursors, (e.g., serotonin), or other pineal products, (e.g., 5-methoxytryptamine). This would depend on the position(s) of the susceptible enzyme(s). For some unknown reason, pineal calcification starts intracellularly. Calcium has been demonstrated in pinealocyte mitochondria. Therefore, it may be a mitochondrial enzyme that is sensitive to the effects of fluoride, e.g., tryptophan-5-hydroxylase. Alternatively, fluoride may affect pinealocyte enzymes which require a divalent co-enzyme because such enzymes are particularly sensitive to fluoride.

Puberty is a developmental stage related to an increase in the hypothalamic-pituitary axis and is triggered by mechanisms which have not yet been fully worked out. Melatonin is a putative neuromodulator involved in the complex process. One well-known hypothesis is that depressed plasma melatonin levels accelerate the onset of puberty. This project offered a unique opportunity to explore this hypothesis because the HF group had depressed plasma melatonin levels during puberty.

The section on the effects of fluoride on the physiological signs of sexual maturity in the gerbil was a preliminary, pilot study. There were not enough subjects to make any firm conclusions so an interpretation of the data is conjectural. However, the results do suggest that the HF females had an accelerated onset of puberty as judged by several indices of pubertal development in rodents. At 7 weeks, the HF females were significantly heavier than the LF females ($p < 0.004$); as heavy as the

HF males and LF males. The ventral gland in the HF female developed significantly earlier than in the LF female ($p < 0.004$). Vaginal opening occurred earlier in the HF female than in the LF female ($p < 0.03$). If there was a difference in male pubertal development between the groups, the elementary methods used in this study were not able to make that distinction.

At 16 weeks, the HF males had a significantly lower mean testes weight than the LF males: 1.10 ± 0.11 *vs.* 1.32 ± 0.18 g, respectively ($p < 0.002$). The reason for this is not clear. At 11½ weeks, the HF males produced significantly less melatonin than at 16 weeks (when their pineal melatonin output reached 'normal' values). Therefore, between 11½ and 16 weeks, the HF males had progressively increasing levels of circulating plasma melatonin. This is unlike the LF males whose circulating plasma melatonin levels were progressively decreasing during the same period of development. (The LF male pineal secreted a constant melatonin output with a uniform rhythm from 11½ to 16 weeks and the increase in body weight, from 73 g at 11½ weeks to 80 g at 16 weeks, would dilute the levels of circulating melatonin). The amplitude, duration and timing of pineal melatonin release, and the phase angle between melatonin rhythms and other reproductive hormones are known to be important in determining the reproductive effects of melatonin. Therefore, the pineals in the HF males relayed an unusual melatonin message to the tissues and organs between 11½ and 16 weeks which may have affected the male reproductive system.

Alternatively, the reason for the reduced rate of urinary aMT6s excretion by the HF group may be that fluoride affected the clearance rate of aMT6s by the kidneys or the rate of melatonin metabolism in the

liver. A recent study (Dunipace *et al*, 1995) investigated the effects of fluoride on kidney and liver function using four groups of rats fed 0, 5, 15 or 50 mg F/L in their drinking water for 18 months. They concluded that fluoride had no adverse physiological or genotoxic effects; did not alter the levels of blood 'wellness' markers of tissue integrity and function; and similar histopathologies in kidney and liver specimens were present across the groups. The rats maintained on water with 50 mg F/L had significantly higher urine urea and creatinine ($p < 0.05$) than the other groups.

However, bone ash from rats maintained on water with 50 mg F/L for 12 weeks contained 5,764 ± 142 mg F/kg (Dunipace *et al*, 1995) which is significantly higher than the fluoride concentration in bone ash from gerbils in the current study which were maintained on food with 37 mg F/kg for 28 weeks, i.e., 2,781 ± 95 mg/kg. The fluoride-concentration in bone is a good index of previous fluoride exposure. Therefore, the fluoride-dose used in this study was not excessive and it is unlikely that the reduced pineal melatonin output by the HF group is due to the effects of fluoride on liver or kidney function.

The daily fluoride-intake by the gerbils in the current study was well tolerated. There was only one mortality (a HF female at 14 weeks). The rationale behind the administration of fluoride to the gerbil pups was to simulate infants bottle-fed on powdered milk formula reconstituted with fluoridated water. These infants receive up to 200 times more fluoride from day 1 than breast-fed infants (Ekstrand *et al*, 1988). The neonatal fluoride-dose to the gerbils (2.3 µg F/g BW/day) was 23 times greater than the estimated threshold fluoride-dose to infants for the development of dental fluorosis. The rat has to receive a 4-5 times

higher fluoride dose in order to achieve a plasma-fluoride level comparable to humans. Dental fluorosis occurred in rats ingesting 25-60 mg F/L in the water (Angmar-Månsson and Whitford, 1984).

The best protection against dental caries is achieved if fluoridation is available from birth (Ripa, 1993). The current view of how fluoride works to prevent the development of dental caries is the maintenance of high fluoride levels in the oral environment (Burt, 1995). Therefore, in order to obtain the maximum reduction of dental caries, the plasma-fluoride levels are increased during infancy and early childhood. Fluoride is now introduced at a much earlier stage of human development than ever before and consequently alters the normal fluoride-pharmacokinetics in infants.

But can one dramatically increase the normal fluoride-intake to infants and get away with it? The safety of the use of fluorides ultimately rests on the assumption that the developing enamel organ is most sensitive to the toxic effects of fluoride. The results from this study suggest that the pinealocytes may be as susceptible to fluoride as the developing enamel organ. The possibility of a species difference between humans and gerbils does not allow the extrapolation of the gerbil data to humans. However, if increased plasma-fluoride levels cause a decline in the levels of circulating melatonin during early human development, significant physiological consequences may have already occurred. Changes in plasma melatonin concentrations are serious functional disturbances because melatonin has many functions in the organism. The pinealogists have not completely unravelled the mechanisms by which the pineal gland performs its tasks in the brain. The neurochemical phenomenon elicited by melatonin in CNS are unclear.

The first step in assessing a health risk by a substance to humans is the identification of its harmful effects on animals. A health risk to humans is assessed using results from human epidemiological studies in conjunction with results from animal studies. The Newburgh-Kingston Study (Schlesinger *et al*, 1956) showed an earlier age of first menarche in girls living in the fluoridated Newburgh than in unfluoridated Kingston. The current animal study indicates that fluoride is associated with an earlier onset of puberty in female gerbils. Furthermore, more research was recommended on the effects of fluoride on animal and human reproduction (USPHS, 1991). This project has contributed new knowledge in this area.

I do not intend to discuss the relative merits of the claims made by the anti-fluoridationists that chronic ingestion of low levels of fluoride has harmful effects on human health, i.e., increases the risk of cancer, affects the immune system, and hastens the aging process. These claims could be associated with the effects of fluoride on the pineal because the gland has been linked to oncogenesis, immunocompetence, and, in recent years, to the process of aging.

In conclusion, the human pineal gland contains the highest concentration of fluoride in the body. Fluoride is associated with depressed pineal melatonin synthesis by prepubertal gerbils and an accelerated onset of sexual maturation in the female gerbil. The results strengthen the hypothesis that the pineal has a role in the timing of the onset of puberty. Whether or not fluoride interferes with pineal function in humans requires further investigation.

APPENDICES

Table A.1 Results of the fluoride levels in aged human pineal glands

Subject	Age years	pineal wet wt mg	mVolts	correln	nmoles F	total F nmoles	ug F	mg F/kg pineal wet
1(i)	79	63	44.5	2.09	124	271	5	82
1(ii)			40.2	2.17	148			
2(i)	78	157	-21.5	3.26	1831	2264	43	274
2(ii)			13.8	2.64	434			
3(i)	78	154	-7.4	3.01	1030	4724	90	585
3(ii)			-38.7	3.57	3694			
4(i)	85	91	3.2	2.82	668	909	17	190
4(ii)			28.2	2.38	241			
5	88	198	-48.7	3.34	2183	2183	41	209
10(i)	100	67	-3.1	2.94	864	1246	24	356
10(ii)			16.9	2.58	382			
12(i)	81	154	-42	3.60	4013	7110	135	875
12(ii)			-35.7	3.49	3097			
13(i)	83	62	78.7	1.49	31	185	4	57
13(ii)			39.1	2.19	154			
14(i)	70	155	-8.7	3.04	1086	3593	68	440
14(ii)			-29.2	3.40	2507			
15(i)	75	56	80.5	1.45	28	42	1	14
15(ii)			99.5	1.12	13			
16(i)	85	75	5.5	2.78	608	700	13	178
16(ii)			51.8	1.96	92			

Pineal No 5

Standards	mVolts		
10 nmoles F	86.55	correlation	-1.0000
100 nmoles F	29.05	intercept	144.55
1000 nmoles F	-29.2	slope	-57.88

Pineal No 12

Standards	mVolts		
1000 nmoles F	-8.3	correlation	1.000
2500 nmoles F	-30.23	intercept	159.9
5000 nmoles F	-47.5	slope	-56.03

All the pineals except Nos. 5 and 12

Standards	mVolts		
1000 nmoles F	-6.6	correlation	-1.0000
10 000 nmoles F	-63.5	intercept	162.58
20 000 nmoles F	-79.8	slope	-56.42

Table A.2(i) Results of the F-contents of human bone (ashed)

sample	bone ash mg	mV	correl-ation	nmoles F	ng F	bone ash mg F/kg	mg F/kg bone ash mean ± SD	CV%
1(i)	1.0018	17.9	2.28	190.5	3619	3612		
1(ii)	1.0018	18	2.28	189.7	3604	3598		
1(iii)	1.0018	18.1	2.28	188.9	3590	3583	3607 ± 33	0.9
1(iv)	1.0018	18	2.28	189.7	3604	3598		
1(v)	1.0018	17.5	2.29	193.5	3677	3670		
1(vi)	1.0018	18.1	2.28	188.9	3590	3583		
5(i)	0.9585	41.3	1.87	74.6	1417	1479		
5(ii)	0.9585	41.4	1.87	74.9	1423	1484		
5(iii)	0.9585	41.3	1.87	75.2	1428	1490	1475 ± 14	0.9
5(iv)	0.9585	41.2	1.88	73.7	1400	1461		
5(v)	0.9585	lost						
5(vi)	0.9585	41.7	1.87	73.7	1400	1461		
12(i)	0.8364	58.9	1.57	37.1	705	843		
12(ii)	0.8364	57.6	1.59	39.1	743	888		
12(iii)	0.8364	59.7	1.56	35.9	683	816	838 ± 30	3.6
12(iv)	0.8364	59.3	1.56	36.5	694	830		
12(v)	0.8364	59.8	1.55	35.8	680	813		
13(i)	0.666	41.2	1.88	75.2	1428	2145		
13(ii)	0.666	41.7	1.87	73.7	1400	2103		
13(iii)	0.666	40.9	1.88	76.1	1446	2171	2123 ± 52	2.4
13(iv)	0.666	41	1.88	75.8	1440	2162		
13(v)	0.666	41.4	1.87	74.6	1417	2128		
13(vi)	0.666	42.6	1.85	71.1	1351	2028		
15(i)	1.515	15.4	2.32	210.4	3998	2639		
15(ii)	1.515	17.3	2.29	195.1	3706	2446		
15(iii)	1.515	14.9	2.33	214.7	4079	2692	2566 ± 94	3.7
15(iv)	0.7576	33.5	2.01	102.2	1942	2564		
15(v)	0.7576	33.3	2.01	103.0	1958	2584		
15(vi)	0.7576	34.4	1.99	98.61	1874	2473		
Blank 1	Blank 1	188.2	-1.50	0.03	0.6			
Blank 2	Blank 2	192.1	-1.57	0.03	0.5			
Standards	10 nmoles F	91.77						
Standards	50 nmoles F	51.8	correlation		-1.0000			
Standards	100 nmoles F	33.5	Regression slope		-57.72			
Standards	500 nmoles F	-6.13	y intercept		149.49			

Table A.2(ii) Results of the F-contents of human bone (ashed)

sample	bone ash mg	mV	correl-ation	nmoles F	ng F	bone ash mg F/kg	mg F/kg bone ash mean ± SD	CV%
2(i)	1.233	-10.1	1.92	83.7	1591	1290		
2(ii)	1.233	-8.8	1.90	79.5	1510	1225		
2(iii)	1.233	-9.8	1.92	82.7	1572	1275	1256 ± 34	2.7
2(iv)	1.233	-9.5	1.91	81.7	1553	1259		
2(v)	1.233	-8.4	1.89	78.2	1487	1206		
2(vi)	1.233	-10	1.92	83.4	1584	1285		
3(i)	0.803	-2.6	1.79	62.1	1180	1469		
3(ii)	0.803	-2.5	1.79	61.9	1176	1464		
3(iii)	0.803	-1	1.77	58.3	1108	1379	1430 ± 37	2.6
3(iv)	0.803	-2.3	1.79	61.4	1166	1452		
3(v)	0.803	-1.4	1.77	59.2	1125	1401		
3(vi)	0.803	-1.6	1.78	59.7	1134	1412		
4(i)	1.361	-4.1	1.82	65.9	1253	921		
4(ii)	1.361	-4.9	1.83	68.1	1294	950		
4(iii)	1.361	-2.5	1.79	61.9	1176	864	910 ± 40	4.4
4(iv)	1.361	-2.3	1.79	61.4	1166	857		
4(v)	1.361	-4.3	1.82	66.5	1263	928		
4(vi)	1.361	-4.7	1.83	67.5	1283	943		
10(i)	1.118	-33.7	2.33	214.0	4065	3638		
10(ii)	1.118	-34.1	2.34	217.4	4130	3696		
10(iii)	1.118	-33.8	2.33	214.8	4082	3652	3711 ± 70	1.9
10(iv)	1.118	-34.2	2.34	218.3	4147	3711		
10(v)	1.118	-34.4	2.34	220.0	4180	3740		
10(vi)	1.118	-35	2.35	225.3	4281	3831		
14(i)	0.887	0.7	1.74	54.5	1035	1167		
14(ii)	0.887	0.3	1.74	55.4	1052	1185		
14(iii)	0.887	0.2	1.74	55.6	1056	1190	1162 ± 34	2.9
14(iv)	0.887	0.2	1.74	55.6	1056	1190		
14(v)	0.887	1.7	1.72	52.4	995	1121		
14(vi)	0.887	1.8	1.72	52.2	991	1117		
16(i)	0.623	-17	2.04	110.1	2093	3360		
16(ii)	0.623	-17.2	2.05	111.0	2109	3387		
16(iii)	0.623	-16.5	2.03	108.0	2052	3294	3329 ± 48	1.4
16(iv)	0.623	-16.9	2.04	109.7	2084	3347		
16(v)	0.623	-16.8	2.04	109.3	2076	3333		
16(vi)	0.623	-16.2	2.03	106.7	2027	3255		
Blank 1		158.3	-0.99	0.1	2			
Blank 2		158.3	-0.99	0.1	2			
Standards	10 nmoles F	43.17						
Standards	50 nmoles F	3	correlation		-1.000			
Standards	100 nmoles F	-14.37	Regression slope		-57.91			
Standards	500 nmoles F	-55.23	y intercept		101.25			

Table A.3 Results of the fluoride concentration of human soft tissue (muscle)

Subject	muscle(wet) mg	mV	correlation	nmoles F	total nmoles F	ng F	mg F/kg muscle (wet)
1(i)	93.3	126.7	0.23	1.7	3.3	62	0.7
1(ii)		128.5	0.19	1.6			
2(i)	101.7	149.5	-0.28	0.5	1.2	23	0.2
2(ii)		143.8	-0.15	0.7			
3(i)	104.2	152.7	-0.36	0.4	1.9	36	0.3
3(ii)		130	0.16	1.4			
4(i)	86.6	129.3	0.18	1.5	1.9	35	0.4
4(ii)		156.2	-0.44	0.4			
5(i)	114.3	101.8	0.80	6.3	8.8	167	1.5
5(ii)		119.7	0.39	2.5			
10(i)	127.5	146.6	-0.22	0.6	1.6	30	0.2
10(ii)		137.4	-0.01	1.0			
12(i)	118.7	136.8	0.00	1.0	1.5	29	0.2
12(ii)		150.6	-0.31	0.5			
13(i)	110.8	123.7	0.30	2.0	4.5	86	0.8
13(ii)		119.3	0.40	2.5			
14(i)	85.5	140	-0.07	0.9	1.4	27	0.3
14(ii)		147.5	-0.24	0.6			
15(i)	112.5	140.1	-0.07	0.9	1.9	36	0.3
15(ii)		136.4	0.01	1.0			
16(i)	127.6	129	0.18	1.5	2.5	48	0.4
16(ii)		136.8	0.00	1.0			

Standards	log	mV		
0.5 nmoles F	-0.3010	147.9	correlation	-0.990
1.0 nmoles F	0	140.9	regression slope	-44
5.0 nmoles F	0.699	105.3	y intercept	137

Table A.4 Results of the calcium concentration in aged human pineal glands

Subject	pineal wt (wet) mg	vol of diffusate ml	length mm	correlation mmol Ca ++	dilution x 4 mmoles Ca++	Ca++ µg	mg Ca/kg pineal (wet)
1	63	6.9	26.0	1.01	4.04	1115	17 784
2	157	2.5	41.0	2.77	8.31	831	5300
3	154	4	115.0	4.47	35.76	5722	37 274
4	91	5.8	75.0	2.90	11.60	2691	29 574
5	198	7.6	6.0	0.23	0.92	280	1412
10	67	4.6	29.5	1.15	4.60	846	12 728
12	154	9.7	62.0	2.41	9.64	3740	24 241
13	62	9.4	5.0	0.19	0.76	286	4639
14	155	8.3	24.0	0.94	3.76	1248	8038
15	56	9	8.0	0.31	1.24	446	7971
16	75	10	15.0	0.58	2.32	928	12 406
blank			1.2	0.05	0.18		

Table A.5 Laboratory results of the F-contents of bone ash from 10-week-old low-F male and 11-week-old low-F female gerbils

SAMPLE	mV	correl-ation	nmoles F	ng F	wt bone (mg) in 1.5 ml 2M HClO4	vol used ul	wt bone ash (mg)	mg F/kg bone ash	mean mg F/kg bone ash
10W LFM 1 (i)	4.3	2.00	100.8	1916	38.0	200	5.07	378	384
10W LFM 1 (ii)	3.5	2.02	104.2	1979	38.0	200	5.07	391	
10W LFM 2 (i)	-0.1	2.08	120.7	2294	39.5	200	5.27	436	436
10W LFM 2 (ii)	-0.2	2.08	121.2	2303	39.5	200	5.27	437	
10W LFM 3 (i)	-8.7	2.23	171.7	3263	52.7	200	7.03	464	464
10W LFM 3 (ii)	-8.7	2.23	171.7	3263	52.7	200	7.03	464	
10W LFM 4 (i)	4.3	2.00	100.8	1916	38.2	200	5.09	376	384
10W LFM 4 (ii)	3.3	2.02	105.0	1996	38.2	200	5.09	392	
10W LFM 5 (i)	4.6	2.00	99.6	1892	30.0	200	4.00	473	468
10W LFM 5 (ii)	5.1	1.99	97.6	1854	30.0	200	4.00	463	
10W LFM 6 (i)	-11.5	2.28	192.6	3660	51.2	200	6.83	536	527
10W LFM 6 (ii)	-10.7	2.27	186.4	3542	51.2	200	6.83	519	
10W LFM 7 (i)	10.5	1.89	78.2	1486	30.1	200	4.01	370	384
10W LFM 7 (ii)	8.8	1.92	83.8	1593	30.1	200	4.01	397	
10W LFM 8 (i)	-4.1	2.15	142.2	2703	41.3	200	5.51	491	492
10W LFM 8 (ii)	-4.2	2.15	142.8	2714	41.3	200	5.51	493	
11W LF F1 (i)	-2.6	2.13	133.8	2541	40.4	200	5.39	472	480
11W LF F1 (ii)	-3.4	2.14	138.2	2626	40.4	200	5.39	488	
11W LF F2 (i)	2.9	2.03	106.8	2029	35.8	200	4.77	425	422
11W LF F2 (ii)	3.2	2.02	105.5	2004	35.8	200	4.77	420	
11W LF F3 (i)	-1.9	2.11	130.0	2470	43.4	200	5.79	427	417
11W LF F3 (ii)	-0.7	2.09	123.7	2351	43.4	200	5.79	406	
11W LF F4 (i)	5.0	1.99	98.0	1861	39.0	200	5.20	358	354
11W LF F4 (ii)	5.6	1.98	95.6	1816	39.0	200	5.20	349	
11W LF F5 (i)	10.0	1.90	79.8	1517	33.4	200	4.45	341	338
11W LF F5 (ii)	10.4	1.90	78.5	1492	33.4	200	4.45	335	
11W LF F6 (i)	-1.2	2.10	126.3	2400	43.8	200	5.84	411	389
11W LF F6 (ii)	1.5	2.05	113.1	2148	43.8	200	5.84	368	
11W LF F7 (i)	13.6	1.84	68.9	1309	38.9	200	5.19	252	250
11W LF F7 (ii)	14.0	1.83	67.8	1287	38.9	200	5.19	248	
11W LF F8 (i)	6.9	1.96	90.6	1722	47.7	200	6.36	271	267
11W LF F8 (ii)	7.5	1.95	88.4	1680	47.7	200	6.36	264	

STANDARDS	mV		
10 nmoles F	60.25	Multiple regression	0.9999
100 nmoles F	5.4	Intercept (c)	116.9
1000 nmoles F	-52.15	x1 (m)	-56.2

Table A.6 Laboratory results of the F-contents of bone ash from 10-week-old high-F male (10W HF M) and 11-week-old high-F female (11W HF F) gerbils.

SAMPLE	mV	correl-ation	nmoles F	ng F	wt bone (mg) in 1.5 ml 2M HClO4	vol used ul	wt bone ash (mg)	mg F/kg bone ash	mean mg F/kg bone ash
10W HF M1 (i)	-15.0	2.27	184.9	3513	59.3	50	1.98	1777	1767
10W HF M1 (ii)	-14.7	2.26	182.7	3471	59.3	50	1.98	1756	
10W HF M2 (i)	-10.3	2.19	153.6	2919	46.8	50	1.56	1871	1814
10W HF M2 (ii)	-8.7	2.16	144.2	2741	46.8	50	1.56	1757	
10W HF M3 (i)	-10.7	2.19	156.1	2965	47.2	50	1.57	1885	1885
10W HF M3 (ii)	lost								
10W HF M4 (i)	-0.6	2.02	104.8	1992	35.7	50	1.19	1674	1725
10W HF M4 (ii)	-2.1	2.05	111.2	2113	35.7	50	1.19	1776	
10W HF M5 (i)	-3.6	2.07	118.0	2242	37.4	50	1.25	1798	1831
10W HF M5 (ii)	-4.5	2.09	122.3	2323	37.4	50	1.25	1863	
10W HF M6 (i)	-8.9	2.16	145.4	2762	38.0	50	1.27	2181	2198
10W HF M6 (ii)	-9.3	2.17	147.7	2806	38.0	50	1.27	2215	
11W HF F1 (i)	-12.0	2.22	164.3	3121	56.3	50	1.88	1663	1663
11W HF F1 (ii)	-12.0	2.22	164.3	3121	56.3	50	1.88	1663	
11W HF F2 (i)	-9.6	2.17	149.5	2840	49.9	50	1.66	1707	1566
11W HF F2 (ii)	-5.0	2.10	124.7	2369	49.9	50	1.66	1424	
11W HF F3 (i)	-1.9	2.04	110.3	2097	45.5	50	1.52	1382	1374
11W HF F3 (ii)	-1.6	2.04	109.1	2072	45.5	50	1.52	1366	
11W HF F4 (i)	12.1	1.80	63.6	1208	26.6	50	0.89	1362	1360
11W HF F4 (ii)	12.2	1.80	63.3	1203	26.6	50	0.89	1357	
11W HF F5 (i)	0.7	2.00	99.6	1893	36.0	50	1.20	1577	1602
11W HF F5 (ii)	-0.1	2.01	102.8	1953	36.0	50	1.20	1628	
11W HF F6 (i)	-5.3	2.10	126.2	2397	46.8	50	1.56	1537	1522
11W HF F6 (ii)	-4.8	2.09	123.7	2350	46.8	50	1.56	1507	
11W HF F7 (i)	11.5	1.81	65.1	1237	35.1	50	1.17	1057	1089
11W HF F7 (ii)	10.0	1.84	69.1	1312	35.1	50	1.17	1121	
11W HF F8 (i)	-9.9	2.18	151.2	2873	51.4	50	1.71	1677	1670
11W HF F8 (ii)	-9.7	2.18	150.0	2851	51.4	50	1.71	1664	

STANDARDS	mV		
10 nmoles F	59.1	Multiple regression	1.000
100 nmoles F	0.5	Intercept (c)	117.5
1000 nmoles F	-57.8	x1 (m)	-58.5

Table A.7 Laboratory results of the F-contents of bone ash from 10-week-old high-F male (10W HFW M) and 11-week-old high-F female (10W HFAW F) gerbils given high-F diet after weaning at 24 days (HFAW).

SAMPLE	mV	correl-ation	nmoles F	ng F	wt bone (mg) in 1.5 ml 2M HClO4	vol used ul	wt bone ash (mg)	mg F/kg bone ash	mean mg F/kg bone ash
10WHF AW M1 (i)	15.9	1.84	69.6	1322	35.4	50	1.18	1120	1150
10WHF AW M1 (ii)	14.6	1.86	73.3	1392	35.4	50	1.18	1180	
10WHF AW M2 (i)	3.2	2.06	115.1	2188	53.5	50	1.78	1227	1205
10WHF AW M2 (ii)	4.1	2.05	111.1	2111	53.5	50	1.78	1184	
10WHF AW M3 (i)	3.0	2.06	116.1	2205	51.7	50	1.72	1280	1306
10WHF AW M3 (ii)	2.0	2.08	120.8	2294	51.7	50	1.72	1331	
10WHF AW M4 (i)	1.7	2.09	122.2	2322	47.2	50	1.57	1476	1398
10WHF AW M4 (ii)	4.5	2.04	109.4	2078	47.2	50	1.57	1321	
10WHF AW M5 (i)	6.7	2.00	100.2	1904	48.8	50	1.63	1171	1211
10WHF AW M5 (ii)	5.0	2.03	107.2	2037	48.8	50	1.63	1252	
10WHF AW M6 (i)	6.2	2.01	102.2	1942	39.7	50	1.32	1468	1442
10WHF AW M6 (ii)	7.1	1.99	98.6	1874	39.7	50	1.32	1416	
10WHF AW M7 (i)	5.7	2.02	104.3	1981	48.6	50	1.62	1223	1213
10WHF AW M7 (ii)	6.1	2.01	102.6	1950	48.6	50	1.62	1204	
10WHF AW M8 (i)	2.9	2.07	116.5	2214	50.1	50	1.67	1326	1364
10WHF AW M8 (ii)	1.5	2.09	123.2	2340	50.1	50	1.67	1401	
11WHF AW F1 (i)	13.7	1.88	75.9	1443	33.9	50	1.13	1277	1292
11WHF AW F1 (ii)	13.1	1.89	77.8	1477	33.9	50	1.13	1307	
11WHF AW F2 (i)	5.0	2.03	107.2	2037	46.3	50	1.54	1320	1333
11WHF AW F2 (ii)	4.5	2.04	109.4	2078	46.3	50	1.54	1346	
11WHF AW F3 (i)	3.5	2.06	113.8	2162	47.6	50	1.59	1363	1354
11WHF AW F3 (ii)	3.8	2.05	112.4	2136	47.6	50	1.59	1346	
11WHF AW F4 (i)	18.3	1.80	63.3	1202	27.4	50	0.91	1316	1293
11WHF AW F4 (ii)	19.2	1.79	61.1	1160	27.4	50	0.91	1270	
11WHF AW F5 (i)	10.9	1.93	84.8	1612	37.9	50	1.26	1276	1276
11WHF AW F5 (ii)	10.9	1.93	84.8	1612	37.9	50	1.26	1276	
BLANK 1	126.2	-0.06	0.9	17					
BLANK 2	125.5	-0.05	0.9	17					
STANDARDS	**mV**								
10 nmoles F	65.2	Multiple regression		1.000					
100 nmoles F	6.00	Intercept (c)		122.9					
1000 nmoles F	-50.9	x1 (m)		-58.1					

Table A. 8 Laboratory results of the F-contents of bone ash from 16-week-old low-F gerbils (male and female mixed).

SAMPLE	mV	correl-ation	nmoles F	ng F	wt bone (mg) in 1.5 ml 2M HClO4	vol used ul	wt bone ash (mg)	mg F/kg bone ash	mean mg F/kg bone ash
16W LF 1 (i)	-26.0	2.43	270.0	5130	58.3	200	7.77	660	667
16W LF 1 (ii)	-26.5	2.44	275.4	5233	58.3	200	7.77	673	
16W LF 2 (i)	-18.6	2.33	212.8	4044	56.0	200	7.47	542	535
16W LF 2 (ii)	-19.4	2.32	207.5	3943	56.0	200	7.47	528	
16W LF 3 (i)	-16.3	2.26	183.4	3485	42.4	200	5.65	616	604
16W LF 3 (ii)	-15.3	2.25	176.3	3349	42.4	200	5.65	592	
16W LF 4 (i)	-23.6	2.39	245.4	4662	67.8	200	9.04	516	517
16W LF 4 (ii)	-23.7	2.39	246.4	4681	67.8	200	9.04	518	
16W LF 5 (i)	-21.7	2.36	227.5	4322	60.6	200	8.08	535	533
16W LF 5 (ii)	-21.5	2.35	225.7	4288	60.6	200	8.08	531	
16W LF 6 (i)	-19.7	2.32	210.0	3991	48.0	200	6.40	624	627
16W LF 6 (ii)	-20.0	2.33	212.6	4039	48.0	200	6.40	631	
16W LF 7 (i)	-2.1	2.02	104.1	1979	33.1	200	4.41	448	481
16W LF 7 (ii)	-5.5	2.08	119.3	2266	33.1	200	4.41	513	
16W LF 8 (i)	-14.9	2.24	173.5	3296	40.8	200	5.44	606	605
16W LF 8 (ii)	-14.8	2.24	172.8	3283	40.8	200	5.44	603	
16W LF 9 (i)	-10.0	2.15	142.7	2711	32.9	200	4.39	618	617
16W LF 9 (ii)	-9.9	2.15	142.1	2700	32.9	200	4.39	616	
16W LF 10 (i)	-15.9	2.26	180.5	3430	37.0	200	4.93	695	691
16W LF 10 (ii)	-15.6	2.25	178.4	3389	37.0	200	4.93	687	
16W LF 11 (i)	11.1	1.79	61.5	1169	22.7	200	3.03	386	389
16W LF 11 (ii)	10.7	1.80	62.5	1188	22.7	200	3.03	393	
16W LF 12 (i)	2.3	1.94	87.4	1661	21.8	200	2.91	571	578
16W LF 12 (ii)	1.7	1.95	89.5	1701	21.8	200	2.91	585	
16W LF 13 (i)	-25.8	2.45	282.6	5370	78.4	200	10.45	514	524
16W LF 13 (ii)	-26.8	2.47	294.0	5586	78.4	200	10.45	534	
16W LF 14 (i)	-15.1	2.27	185.4	3523	36.7	200	4.89	720	733
16W LF 14 (ii)	-16.0	2.28	192.1	3650	36.7	200	4.89	746	
16W LF 15 (i)	-16.6	2.29	196.7	3737	45.7	200	6.09	613	616
16W LF 15 (ii)	-16.8	2.30	198.3	3767	45.7	200	6.09	618	
16W LF 16 (i)	-7.5	2.14	137.4	2611	35.1	200	4.68	558	542
16W LF 16 (ii)	-6.0	2.11	129.5	2461	35.1	200	4.68	526	

STANDARDS	mV
10 nmole F	56.8
100 nmole F	-1.27
1000 nmole F	-58.7

ASSAY 1	
Multiple regression	1.0000
Intercept (c)	114.5
x1 (m)	-57.77

STANDARDS	mV
10 nmole F	59.1
100 nmole F	0.5
1000 nmole F	-57.8

ASSAY 2	
Multiple regression	1.0000
Intercept (c)	117.5
x1 (m)	-58.4

Table A.9 Laboratory results of the F-contents of bone ash from 16-week-old high-F male (16W HF M) and female (16W HF F) gerbils.

SAMPLE	mV	correl-ation	nmoles F	ng F	wt bone (mg) in 1.5 ml 2M HClO4	vol used ul	wt bone ash (mg)	mg F/kg bone ash	mean mg F/kg bone ash
16W HF M1 (i)	-11.4	2.18	150.9	2867	33.7	50	1.12	2552	2527
16W HF M1 (ii)	-10.9	2.17	147.9	2810	33.7	50	1.12	2502	
16W HF M2 (i)	-13.5	2.21	164.1	3117	36.4	50	1.21	2569	2637
16W HF M2 (ii)	-14.8	2.24	172.8	3283	36.4	50	1.21	2706	
16W HF M3 (i)	-18.6	2.30	201.0	3820	43.0	50	1.43	2665	2725
16W HF M3 (ii)	-19.7	2.32	210.0	3991	43.0	50	1.43	2784	
16W HF M4 (i)	-25.3	2.42	262.6	4989	68.2	50	2.27	2195	2203
16W HF M4 (ii)	-25.5	2.42	264.7	5029	68.2	50	2.27	2212	
16W HF M5 (i)	-23.6	2.39	245.4	4662	57.0	50	1.90	2454	2449
16W HF M5 (ii)	-22.1	2.39	244.5	4646	57.0	50	1.90	2445	
16W HF M6 (i)	-17.0	2.28	188.6	3584	49.0	50	1.63	2194	2190
16W HF M6 (ii)	-16.9	2.27	187.9	3569	49.0	50	1.63	2185	
16W HF M7 (i)	-25.2	2.42	261.5	4969	63.0	50	2.10	2366	2357
16W HF M7 (ii)	-25.0	2.41	259.5	4930	63.0	50	2.10	2347	
16W HF M8 (i)	-20.3	2.33	215.1	4087	43.5	50	1.45	2819	2597
16W HF M8 (ii)	-16.0	2.26	181.2	3444	43.5	50	1.45	2375	
16W HF F 1 (i)	-31.8	2.53	340.2	6464	64.3	50	2.14	3016	2928
16W HF F 1 (ii)	-30.3	2.51	320.5	6089	64.3	50	2.14	2841	
16W HF F2 (i)	-18.0	2.29	196.3	3729	66.4	50	2.21	1685	1762
16W HF F2 (ii)	-20.2	2.33	214.3	4071	66.4	50	2.21	1839	
16W HF F3 (i)	-31.2	2.52	332.2	6311	63.9	50	2.13	2963	3030
16W HF F3 (ii)	-32.3	2.54	347.1	6594	63.9	50	2.13	3096	
16W HF F4 (i)	-36.4	2.61	408.7	7765	82.6	50	2.75	2820	2744
16W HF F4 (ii)	-35.0	2.59	386.5	7344	82.6	50	2.75	2667	
16W HF F5 (i)	-23.3	2.38	242.5	4607	48.1	50	1.60	2873	2902
16W HF F5 (ii)	-23.8	2.39	247.3	4699	48.1	50	1.60	2931	
16W HF F6 (i)	-35.4	2.59	392.7	7462	64.6	50	2.15	3465	3458
16W HF F6 (ii)	-35.3	2.59	391.2	7432	64.6	50	2.15	3451	
16W HF F7 (i)	-25.5	2.42	264.7	5029	57.1	50	1.90	2642	2616
16W HF F7 (ii)	-25.0	2.41	259.5	4930	57.1	50	1.90	2590	
16W HF F8 (i)	-15.9	2.26	180.5	3430	48.7	50	1.62	2113	2096
16W HF F8 (ii)	-15.5	2.25	177.7	3376	48.7	50	1.62	2079	

STANDARDS	mV		
10 nmoles F	56.8	Multiple regression	1.0000
100 nmoles F	-1.27	Intercept (c)	114.5
1000 nmoles F	-58.7	x1 (m)	-57.77

Table A.10 (i) Laboratory results of the F-contents of bone ash from 28-week-old low-F male (28W LF M) gerbils .

SAMPLE	mV	correl-ation	nmoles F	total nmoles F	ng F	wt bone ash (mg)	mg F/kg bone ash
28W LF M1 (i)	-16.8	2.40	252.1	1301	24 717	37.5	659
28W LF M1 (ii)	-17.6	2.42	260.2				
28W LF M1 (iii)	-16.8	2.40	252.1				
28W LF M1 (iv)	-19.3	2.44	278.3				
28W LF M1 (v)	-17.4	2.41	258.2				
28W LF M2 (i)	-16.7	2.40	251.1	1295	24 610	43.5	566
28W LF M2 (ii)	-17.6	2.42	260.2				
28W LF M2 (iii)	-18.5	2.43	269.7				
28W LF M2 (iv)	-20.3	2.46	289.6				
28W LF M2 (v)	-13.9	2.35	224.7				
28W LF M3 (i)	-13.5	2.34	221.2	1081	20 531	34.4	597
28W LF M3 (ii)	-14.8	2.37	232.9				
28W LF M3 (iii)	-14.5	2.36	230.1				
28W LF M3 (iv)	-16.1	2.39	245.2				
28W LF M3 (v)	-3.9	2.18	151.2				
28W LF M4 (i)	-23.4	2.52	327.5	1645	31 256	48	651
28W LF M4 (ii)	-23.0	2.51	322.3				
28W LF M4 (iii)	-22.6	2.50	317.2				
28W LF M4 (iv)	-23.1	2.51	323.6				
28W LF M4 (v)	-25.4	2.55	354.5				
28W LF M5 (i)	-20.9	2.47	296.6	1551	29 460	54.6	540
28W LF M5 (ii)	-21.3	2.48	301.3				
28W LF M5 (iii)	-21.9	2.49	308.6				
28W LF M5 (iv)	-21.7	2.49	306.1				
28W LF M5 (v)	-24.2	2.53	338.0				
28W LF M6 (i)	-16.1	2.39	245.2	1199	22 775	45.1	505
28W LF M6 (ii)	-20.1	2.46	287.3				
28W LF M6 (iii)	-16.5	2.40	249.1				
28W LF M6 (iv)	-15.1	2.37	235.7				
28W LF M6 (v)	-8.5	2.26	181.4				

Table A.10 (ii) Laboratory results of the F-contents of bone ash from 28-week-old low-F female (28W LF F) gerbils .

SAMPLE	mV	correl-ation	nmoles F	total nmoles F	ng F	wt bone ash (mg)	mg F/kg bone ash
28W LF F1 (i)	-23.2	2.51	324.9	1550	29 456	51.9	568
28W LF F1 (ii)	-23.1	2.51	323.6				
28W LF F1 (iii)	-23.2	2.51	324.9				
28W LF F1 (iv)	-20.7	2.47	294.2				
28W LF F1 (v)	-19.7	2.45	282.8				
28W LF F2 (i)	-16.9	2.40	253.1	1247	23 691	34.4	689
28W LF F2 (ii)	-15.3	2.38	237.5				
28W LF F2 (iii)	-16.9	2.40	253.1				
28W LF F2 (iv)	-16.9	2.40	253.1				
28W LF F2 (v)	-16.6	2.40	250.1				
28W LF F3 (i)	-27.1	2.58	379.2	1963	37 301	56.2	664
28W LF F3 (ii)	-27.5	2.59	385.2				
28W LF F3 (iii)	-27.3	2.58	382.2				
28W LF F3 (iv)	-26.4	2.57	368.8				
28W LF F3 (v)	-31.3	2.65	447.8				
28W LF F4 (i)	-34.2	2.70	502.4	2545	48 351	48.8	991
28W LF F4 (ii)	-34.3	2.70	504.4				
28W LF F4 (iii)	-33.1	2.68	480.9				
28W LF F4 (iv)	-34.2	2.70	502.4				
28W LF F4 (v)	-36.7	2.74	554.7				
28W LF F5 (i)	-36.6	2.74	552.5	2844	54 029	65	831
28W LF F5 (ii)	-36.5	2.74	550.3				
28W LF F5 (iii)	-36.6	2.74	552.5				
28W LF F5 (iv)	-37.1	2.75	563.6				
28W LF F5 (v)	-39.7	2.80	624.7				
28W LF F6 (i)	-30.2	2.63	428.7	2236	42 492	57.5	739
28W LF F6 (ii)	-31.6	2.66	453.2				
28W LF F6 (iii)	-30.8	2.64	439.0				
28W LF F6 (iv)	-30.2	2.63	428.7				
28W LF F6 (v)	-33.4	2.69	486.7				
28W LF F7 (i)	-27.1	2.58	379.2	1894	35 990	59.2	608
28W LF F7 (ii)	-27.1	2.58	379.2				
28W LF F7 (iii)	-24.8	2.54	346.1				
28W LF F7 (iv)	-27.0	2.58	377.7				
28W LF F7 (v)	-29.2	2.61	412.1				
BLANK	136.6	-0.24	0.6	0.6	11		
BLANK	129.4	-0.11	0.8	0.8	15		
BLANK	142.0	-0.33	0.5	0.5	10		
BLANK	148.0	-0.43	0.4	0.4	8		
STANDARDS	**mV**						
10 nmole F	64.6	Multiple Regression			1.000		
100 nmole F	6.6	Intercept			122.7		
1000 nmole F	-51.6	x1			-58.1		

Table A.11 Laboratory results of the F-contents of bone ash from 28-week-old high-F male (28W HF M) and female (28W HF F) gerbils.

SAMPLE	mV	correl-ation	nmoles F	ng F	wt bone (mg) in 1.5 ml 2M HClO4	vol used ul	wt bone ash (mg)	mg F/kg bone ash	mean mg F/kg bone ash
28W HF M1 (i)	-37.0	2.63	428.6	8144	46.5	100	3.10	2627	2648
28W HF M1 (ii)	-37.4	2.64	435.6	8276	46.5	100	3.10	2670	
28W HF M2 (i)	-45.1	2.77	593.0	11266	61.0	100	4.07	2770	2833
28W HF M2 (ii)	-46.2	2.79	619.7	11774	61.0	100	4.07	2895	
28W HF M3 (i)	-43.3	2.74	551.7	10483	58.2	100	3.88	2702	2713
28W HF M3 (ii)	-43.5	2.75	556.2	10567	58.2	100	3.88	2723	
28W HF M4 (i)	-33.3	2.57	369.6	7022	36.8	100	2.45	2862	2868
28W HF M4 (ii)	-33.4	2.57	371.1	7050	36.8	100	2.45	2874	
28W HF M5 (i)	-39.0	2.67	464.4	8824	46.3	100	3.09	2859	2842
28W HF M5 (ii)	-38.7	2.66	458.9	8718	46.3	100	3.09	2825	
28W HF F1 (i)	-44.5	2.76	578.9	10999	54.2	100	3.61	3044	3032
28W HF F1 (i)	-44.3	2.76	574.3	10911	54.2	100	3.61	3020	
28W HF F2 (i)	-46.7	2.80	632.2	12012	59.1	100	3.94	3049	2977
28W HF F2 (ii)	-45.5	2.78	602.5	11448	59.1	100	3.94	2906	
28W HF F3 (i)	-32.3	2.56	365.1	6938	76.0	50	2.53	2739	2695
28W HF F3 (ii)	-49.5	2.85	707.3	13438	76.0	100	5.07	2652	
28W HF F4 (i)	-43.6	2.75	558.4	10609	54.5	100	3.63	2920	2908
28W HF F4 (ii)	-43.4	2.74	553.9	10525	54.5	100	3.63	2897	
28W HF F5 (i)	-46.9	2.80	637.3	12109	58.4	100	3.89	3110	3129
28W HF F5 (ii)	-47.2	2.81	645.0	12255	58.4	100	3.89	3148	
28W HF F6 (i)	-40.6	2.69	495.2	9408	52.8	100	3.52	2673	2605
28W HF F6 (ii)	-39.3	2.67	470.0	8930	52.8	100	3.52	2537	
28W HF F7 (i)	-35.7	2.61	406.9	7731	44.2	100	2.95	2624	2603
28W HF F7 (ii)	-35.3	2.60	400.4	7608	44.2	100	2.95	2582	
28W HF F8 (i)	-32.7	2.56	360.8	6856	44.3	100	2.95	2321	2317
28W HF F8 (ii)	-32.6	2.56	359.4	6828	44.3	100	2.95	2312	

STANDARDS	mV		
10 nmole F	57.1	Multiple Regression	1.0000
100 nmole F	-1.15	Intercept	114.3
1000 nmole F	-57.9	x1	-57.5

Table B.1 Laboratory results of the intra-assay and inter-assay coefficients of variation for RIAs for pineal melatonin.

(i) Intra-assay coefficients of variation for melatonin

	MT pg/0.5 ml		MT pg/0.5 ml		MT pg/0.5 ml
Low QC	39	Medium QC	108	High QC	342
Low QC	41	Medium QC	118	High QC	343
Low QC	37	Medium QC	125	High QC	322
Low QC	37	Medium QC	121	High QC	304
Low QC	36	Medium QC	125	High QC	302
Low QC	38	Medium QC	114	High QC	297
Low QC	41	Medium QC	119	High QC	304
Low QC	34	Medium QC	120	High QC	341
Low QC	30	Medium QC	110		
		Medium QC	114		
MEAN	37.2	MEAN	117	MEAN	319
STDEV	3.6	STDEV	5.7	STDEV	20.1
CV%	9.6	CV%	4.9	CV%	6.3

(ii) Inter-assay coefficients of variation for melatonin

	Low	Medium	High
16W LFNM	29.6	90.2	261
16W LF and 16W HF	24.3	86.1	299
MEAN	27.0	88.2	280
STDEV	3.7	2.9	26.9
CV%	13.9	3.3	9.6

Table B.2 Laboratory results of RIA for pineal MT from low-F gerbils aged 16-weeks: non-monitored group. Data expressed as pg MT/pineal gland

QCs: 29.6, 90.2, 261

sample	time	volume µl	vol used µl	[MT] pg/0.5 ml	total MT pg/0.5 ml	total MT pg/gland
16W LFNM F4	16:00	1100	250	32.9	66	145
16W LFNM F8	16:00	1200	350	41.3	59	142
16W LFNM F9	16:00	1050	350	35.6	51	107
16W LFNM M8	16:00	1000	350	23.3	33	67
16W LFNM F5	10:00	900	300	34.9	58	105
16W LFNM F7	10:00	950	350	27.7	40	75
16W LFNM M6	10:00	800	250	30.0	60	96
16W LFNM M7	10:00	1100	400	29.9	37	82
16W LFNM F1	22:00	1000	200	23.2	58	116
16W LFNM F2	22:00	1000	200	15.2	38	76
16W LFNM F3	22:00	900	200	22.4	56	101
16W LFNM M1	22:00	800	200	20.6	52	82
16W LFNM M2	22:00	900	200	20.6	52	93
16W LFNM M3	22:00	900	200	35.8	90	161
16W LFNM F10	04:00	950	200	149.4	374	710
16W LFNM F11	04:00	950	200	319.6	799	1518
16W LFNM F12	04:00	1030	200	72.0	180	342
16W LFNM M9	04:00	1200	200	45.5	114	273
16W LFNM M10	04:00	900	200	46.4	116	209
16W LFNM M11	04:00	1100	200	57.5	144	316

Table B.3 Laboratory results of RIA for pineal MT from high- and low-F gerbils aged 16 weeks. Data expressed as pg MT/pineal gland

QCs: 24.3, 86.1, 299

sample	time	volume µl	vol used µl	[MT] pg/0.5 ml	total MT pg/0.5 ml	total MT pg/gland
16W LF F1	16:00	1000	300	58.1	97	194
16W LF F5	16:00	1000	200	13.8	35	69
16W LF F9	16:00	1000	300	31.2	52	104
16W LF M1	16:00	1000	300	30.1	50	100
16W LF M5	16:00	1000	300	24.7	41	82
16W LF M9	16:00	1000	300	25.0	42	83
16W HF F7	16:00	1000	300	52.6	88	175
16W HF F8	16:00	1000	300	42.0	70	140
16W HF M2	16:00	1000	300	19.3	32	64
16W HF M3	16:00	1000	300	18.1	30	60
16W HF F2	10:00	1200	300	40.0	67	160
16W HF F5	10:00	1000	300	27.4	46	91
16W HF F6	10:00	1000	300	31.7	53	106
16W HF M1	10:00	1000	300	13.3	22	44
16W HF M6	10:00	1000	300	24.0	40	80
16W HF M9	10:00	1000	300	13.0	22	43
16W LF M10	10:00	1000	300	19.7	33	66
16W LF M11	10:00	1000	300	57.9	97	193
16W LF M12	10:00	1200	300	20.0	33	80
16W LF F10	10:00	1000	300	36.5	61	122
16W LF F11	10:00	1000	300	29.6	49	99
16W LF F2	22:00	1000	200	37.2	93	186
16W LF F3	22:00	1000	200	29.2	73	146
16W LF F4	22:00	1000	200	75.3	188	377
16W LF M2	22:00	1000	200	16.8	42	84
16W LF M3	22:00	1000	200	27.5	69	138
16W LF M4	22:00	1000	200	15.3	38	77
16W LF F6	04:00	1000	200	129.6	324	648
16W LF F7	04:00	1000	200	241.0	603	1205
16W LF F8	04:00	1200	200	71.5	179	429
16W LF M6	04:00	1000	200	76	190	380
16W LF M7	04:00	1000	200	247.7	619	1239
16W LF M8	04:00	1000	200	268.5	671	1343
16W HF F1	04:00	1000	200	121.9	305	610
16W HF F3	04:00	1000	200	147.6	369	738
16W HF F4	04:00	1000	200	76.0	190	380
16W HF M10	04:00	1000	200	86.5	216	433
16W HF M11	04:00	1000	200	72.6	182	363
16W HF M12	04:00	1200	200	194.7	487	1168

Table B.4 Inter-assay variability of RIAs for urinary aMT6s excreted by 16-week-old gerbils (used for correlation between total 24-h aMT6s excretion and pineal MT contents at 04:00).

Assay	LOW	MEDIUM	HIGH
16W LF F (1-8)	3.5	14.3	19.5
16W HF M (1-3)	3.8	14.9	25.4
16W HF M (4-9)	3.7	14.5	25.3
16W HF M (9-12)	3.9	14.9	21.1
16W LF M (1-4)	3.7	13.5	23.5
16W LF M (5-8)	4.0	14.1	23.2
16W LF M (9-12)	3.5	13.6	26.2
16W HF F (1-4)	3.6	13.2	23.1
16W HF F (5-8)	3.8	13.7	22.5
16W HF F (9-12)	3.6	12.6	22.1
16W LFNM F (1-4)	2.6	14.4	26.2
16W LFNM F (5-8)	3.4	14.9	22.3
16W LFNM F (9-12)	2.6	13.8	24.4
16W LFNM M (1-4)	3.4	14.8	26.9
16W LFNM M (5-8)	2.8		27.6
16W LFNM M (9-11)	2.1	16	25.3
mean	3.4	14.2	24.0
SD	0.5	0.8	2.2
CV%	16.3	6.0	9.3

Table B.5 Results of melatonin levels at 0400 in gerbil pineal with corresponding total 24-h urinary aMT6s levels

sample	0400 MT pg/pineal	aMT6s pg/g BW/24-h
16W LF F6	648	412
16W LF F7	1205	554
16W LF F8	429	315
16W LF M6	380	181
16W LF M7	1239	502
16W LF M8	1343	617
16W HF F1	610	449
16W HF F3	738	481
16W HF F4	380	380
16W HF M10	433	388
16W HF M11	363	302
16W HF M12	1168	475
16W LFNM F10	710	447
16W LFNM F11	1518	459
16W LFNM F12	342	337
16W LFNM M9	273	439
16W LFNM M10	209	400
16W LFNM M11	316	343
mean	683	416
SD	421	100
SEM	99	24
Pearson's coefficient	0.73	

Table C.1 Laboratory results of intra-assay coefficients of variation using dilutions of QCs

	1:50		1:25	1:10	
Low QC	aMT6s print out	aMT6s ng/ml	aMT6s ng/ml	aMT6s print out	aMT6s ng/ml
1	1.752	3.5	3.6	8.142	3.3
2	1.617	3.2	3.6	8.920	3.6
3	1.809	3.6	3.3	8.889	3.6
4	1.845	3.7	3.7	9.141	3.7
5	1.700	3.4	3.5	8.343	3.3
6	1.727	3.5	3.4	8.712	3.5
7	1.712	3.4	3.4	8.372	3.3
8	1.814	3.6	3.8	8.454	3.4
mean	1.747	3.5	3.5	8.622	3.4
SD	0.07	0.15	0.15	0.35	0.14
CV%	4.3	4.3	4.3	4.0	4.0
Medium QC	aMT6s print out	aMT6s ng/ml	aMT6s ng/ml	aMT6s print out	aMT6s ng/ml
1	5.880	11.8	11.6	27.380	11.0
2	5.977	12.0	13.6	29.386	11.8
3	6.365	12.7	11.5	28.658	11.5
4	5.987	12.0	12.1	28.864	11.5
5	5.650	11.3	12.1	32.893	13.2
6	6.634	13.3	12.8	26.088	10.4
7	6.617	13.2	10.8	26.726	10.7
8	5.784	11.6	13.6		
mean	6.112	12.2	12.3	28.571	11.4
SD	0.38	0.76	1.00	2.25	0.90
CV%	6.2	6.2	8.2	7.9	7.9
High QC	aMT6s print out	aMT6s ng/ml	aMT6s ng/ml		
1	13.118	26.2	24.1		
2	13.579	27.2	25.3		
3	11.434	22.9	24.3		
4	12.301	24.6	26.2		
5	12.393	24.8	23.9		
6	12.352	24.7	22.1		
7	13.324	26.6			
8	11.520	23.0			
mean	12.503	25.0	24.3		
SD	0.79	1.58	1.38		
CV%	6.3	6.3	5.7		

Table C.2 The inter-assay coefficients of variation for RIAs for urinary aMT6s excreted by the gerbils in the longitudinal study

ASSAY	LOW	MEDIUM	HIGH
7W HF and LF males	3.6	14.2	24.1
7W HF and LF females	4.0	12.7	23.1
9W HF and LF males	3.6	14.5	25.9
9W HF and LF females	3.9	13.8	20.3
11½W LF females (1-4)	3.6	14.2	22.3
11½W LF females (5-8)	3.5	13.1	19.8
11½W LF females (9-12)	3.6	13.3	21.2
11½W LF males (1-4)	3.5	13.4	22.1
11½W LF males (5-8)	3.7	14.7	24.7
11½W LF males (9-12)	3.4	13.3	25.5
11½W HF females (1-4)	3.7	12.5	21.0
11½W HF females (5 -8)	3.4	12.7	26.0
11½W HF females (9-12)	3.7	14.1	22.3
11½W HF males (1 - 4)	3.9	14.1	26.1
11½W HF males (5 -8)	3.7	13.2	22.5
11½W HF males (9-12)	3.5	13.2	20.0
16W LF females (1-8)	3.5	14.3	19.5
16W HF males (1-3)	3.8	14.9	25.4
16W HF males (4-9)	3.7	14.5	25.3
16W HF males (9-12)	3.9	14.9	21.1
16W LF males (5-8)	3.7	13.5	23.5
16W LF males (9-12)	4.0	14.1	23.2
16W LF males (1-4)	3.5	13.6	26.2
16W HF females (1-4)	3.6	13.2	23.1
16W HF females (5-8)	3.8	13.7	22.5
16W HF females (9-12)	3.6	12.6	22.1
mean	3.7	13.7	23.0
SD	0.2	0.7	2.1
CV%	4.7	5.2	9.2

Table C.3 The inter-assay coefficients of variation for all RIAs for urinary aMT6s excreted by gerbils.

DESCRIPTION of ASSAY	LOW	MEDIUM	HIGH
7W HF and LF males	3.6	14.2	24.1
7W HF and LF females	4.0	12.7	23.1
9W HF and LF males	3.6	14.5	25.9
9W HF and LF females	3.9	13.8	20.3
11½W LF females (1-4)	3.6	14.2	22.3
11½W LF females (5-8)	3.5	13.1	19.8
11½W LF females (9-12)	3.6	13.3	21.2
11½W LF males (1-4)	3.5	13.4	22.1
11½W LF males (5-8)	3.7	14.7	24.7
11½W LF males (9-12)	3.4	13.3	25.5
11½W HF females (1 - 4)	3.7	12.5	21.0
11½W HF females (5 -8)	3.4	12.7	26.0
11½W HF females (9 - 12)	3.7	14.1	22.3
11½W HF males (1 - 4)	3.9	14.1	26.1
11½W HF males (5 -8)	3.7	13.2	22.5
11½W HF males (9 - 12)	3.5	13.2	20.0
16W LF females (1-8)	3.5	14.3	19.5
16W HF males (1-3)	3.8	14.9	25.4
16W HF males (4-9)	3.7	14.5	25.3
16W HF males (9-12)	3.9	14.9	21.1
16W LF males (5-8)	3.7	13.5	23.5
16W LF males (9-12)	4.0	14.1	23.2
16W LF males (1-4)	3.5	13.6	26.2
16W HF females (1-4)	3.6	13.2	23.1
16W HF females (5-8)	3.8	13.7	22.5
16W HF females (9-12)	3.6	12.6	22.1
HF pubertal males (24-h)	3.8	12.9	25.3
HF pubertal females (24-h)	3.5	13	24.3
LF pubertal males (24-h)	3.3	13.5	25.3
28W LF and HF females	4.1	14.6	21.7
28W LF and HF males	3.6	13.1	21.3
16W LF and HF females	3.8	13.1	24.7
Repeats	4.0	14.9	22.3
MEAN	3.7	13.7	23.1
SD	0.2	0.7	2.0
CV%	5.3	5.3	8.8

Table C.4 The inter-assay coefficients of variation for RIAs for urinary aMT6s excreted by gerbils at 11½ and 16 weeks.

ASSAY	LOW	MEDIUM	HIGH
11½W LF females (1-4)	3.6	14.2	22.3
11½W LF females (9-12)	3.5	13.1	19.8
11½W LF females (5-8)	3.6	13.3	21.2
11½W LF males (1-4)	3.5	13.4	22.1
11½W LF males (5-8)	3.7	14.7	24.7
11½W LF males (9-12)	3.4	13.3	25.5
11½W HF females (1-4)	3.7	12.5	21.0
11½W HF females (5-8)	3.4	12.7	26.0
11½W HF females (9-12)	3.7	14.1	22.3
11½W HF males (1-4)	3.9	14.1	26.1
11½W HF males (5-8)	3.7	13.2	22.5
11½W HF males (9-12)	3.5	13.2	20.0
16W LF females (1-8)	3.5	14.3	19.5
16W HF males (1-3)	3.8	14.9	25.4
16W HF males (4-8)	3.7	14.5	25.3
16W HF males (9-12)	3.9	14.9	21.1
16W LF males (1-4)	3.7	13.5	23.5
16W LF males (5-8)	4.0	14.1	23.2
16W LF females (9 - 12)	3.5	13.6	26.2
16W HF females (1-4)	3.6	13.2	23.1
16W HF females (5-8)	3.8	13.7	22.5
16W HF females (9-12)	3.6	12.6	22.1
MEAN	3.7	13.7	23.0
SD	0.2	0.7	2.1
CV%	4.5	5.2	9.2

Table C.5 Results of coefficients of variations between levels of aMT6s in urine collected in day 1 and day 2 by 7-week-old gerbils

	day 1	day 2	mean	SD	CV%
7 week low-F females	35.9	38.0	37.0	1.5	4.0
	19.4	24.3	21.9	3.5	15.9
	21.7	22.1	21.9	0.3	1.3
	31.9	40.4	36.2	6.0	16.6
	35.7	38.3	37.0	1.8	5.0
	27.7	29.3	28.5	1.1	4.0
	27.2	27.9	27.6	0.5	1.8
	23.4	27.6	25.5	3.0	11.6
	18.8	32.9	25.9	10.0	38.6
	18.2	23.1	20.7	3.5	16.8
	14.1	21.3	17.7	5.1	28.8
	18.9	21.4	20.2	1.8	8.8
7 week high-F females	28.5	26.6	27.6	1.3	4.9
	12.1	19.0	15.6	4.9	31.4
	26.5	24.7	25.6	1.3	5.0
	15.1	14.7	14.9	0.3	1.9
	11.2	13.2	12.2	1.4	11.6
	12.8	16.6	14.7	2.7	18.3
	11.5	18.2	14.9	4.7	31.9
	14.4	15.7	15.1	0.9	6.1
	13.1	15.9	14.5	2.0	13.7
	24.4	30.5	27.5	4.3	15.7
	17.3	17.8	17.6	0.4	2.0
	18.2	16.6	17.4	1.1	6.5
7 week low-F males	31.3	35.9	33.6	3.3	9.8
	27.0	29.0	28.0	1.4	5.0
	36.2	39.6	37.9	2.4	6.3
	38.6	34.3	36.5	3.0	8.3
	38.8	44.1	41.5	3.8	9.1
	24.0	26.7	25.4	1.9	7.4
	40.3	40.1	40.2	0.2	0.4
	28.7	45.1	36.9	11.6	31.4
	22.4	22.0	22.2	0.3	1.3
	19.3	27.3	23.3	5.7	24.4
	21.0	19.0	20.0	1.4	7.3
	18.6	26.6	22.6	5.7	25.0
7 week high-F males	8.9	15.1	12.0	4.4	36.5
	25.1	32.0	28.5	4.9	17.3
	11.4	15.6	13.5	3.0	22.0
	12.0	17.3	14.6	3.8	13.2
	6.9	31.9	19.4	17.7	91.2
	13.3	16.1	14.7	1.9	13.2
	15.8	15.6	15.7	0.1	0.8
	15.7	17.4	16.5	1.2	7.1
	15.0	14.6	14.8	0.2	1.7
	14.2	18.2	16.2	2.8	17.3
	14.9	17.4	16.2	1.8	10.9
	12.7	17.1	14.9	3.1	21.1

Table C.6 Results of coefficients of variations between levels of aMT6s in urine collected in day 1 and day 2 by 9-week-old gerbils

	day 1	day 2	mean	SD	CV%
9 week low-F females	29.3	36.5	32.9	5.0	15.3
	19.6	22.9	21.2	2.3	10.9
	22.6	20.5	21.6	1.5	6.9
	34.5	34.3	34.4	0.1	0.4
	33.3	33.1	33.2	0.1	0.4
	29.7	25.8	29.7	2.8	9.4
	27.9	27.7	27.8	0.2	0.7
	22.7	32.1	27.4	6.7	24.3
	23.6	22.1	22.9	1.0	4.6
	22.9	22.7	22.8	0.2	0.8
	20.2	25.3	22.7	3.6	15.7
	12.5	12.0	12.3	0.4	3.1
9 week high-F females	27.7	26.9	27.3	0.6	2.1
	13.0	22.1	17.6	6.4	36.5
	23.2	36.6	29.9	9.5	31.7
	17.2	24.9	21.0	5.5	26.0
	11.9	12.1	12.0	0.1	1.0
	13.0	18.7	15.9	4.0	25.3
	13.1	18.3	15.7	3.7	23.4
	21.3	38.0	29.6	11.8	39.9
	13.8	12.6	13.2	0.9	6.5
	17.8	29.8	23.8	8.5	35.9
	16.4	22.6	19.5	4.4	22.5
	14.9	18.5	16.7	2.5	15.1
9 week low-F males	21.3	27.6	24.4	4.5	18.4
	23.9	26.6	25.3	1.9	7.5
	34.2	38.1	36.1	2.8	7.7
	33.8	39.8	36.8	4.3	11.7
	28.3	32.9	30.6	3.3	10.7
	27.0	25.5	26.3	1.1	4.0
	29.3	34.0	31.6	3.3	10.5
	42.2	43.3	42.8	0.8	1.8
	18.6	23.9	21.2	3.7	17.4
	17.4	19.3	18.4	1.3	7.3
	16.7	23.1	19.9	4.5	22.8
	20.4	23.5	21.9	2.2	10.1
9 week high-F males	19.6	13.3	16.5	4.4	26.9
	20.2	41.0	30.6	14.7	48.0
	11.4	15.2	13.3	2.7	20.1
	12.0	19.3	15.7	5.2	33.1
	15.7	25.6	20.7	7.0	33.9
	15.2	16.1	15.6	0.6	4.0
	18.1	19.9	19.0	1.3	6.7
	16.6	22.9	19.7	4.5	22.8
	13.7	17.7	15.7	2.8	17.9
	15.1	20.6	17.9	3.8	21.5
	22.4	24.9	23.7	1.7	7.3
	21.6	26.2	23.9	3.3	13.6

Table C.7 Results of coefficients of variations between levels of aMT6s in urine collected in day 1 and day 2 by 11½-week-old gerbils

	day 1	day 2	mean	SD	CV%
11½ week low-F females	34.6	41.2	37.9	4.7	12.3
	22.5	29.9	26.2	5.2	20.0
	20.6	19.9	20.3	0.5	2.4
	42.5	40.9	41.7	1.1	2.7
	30.3	33.1	31.7	2.0	6.2
	24.3	29.7	27.0	3.8	14.1
	23.3	29.2	26.3	4.2	15.9
	23.5	26.1	24.8	1.8	7.4
	26.0	26.2	26.1	0.1	0.5
	22.9	29.7	26.3	4.8	18.3
	28.4	30.6	29.5	1.6	5.4
	15.0	15.5	15.3	0.4	2.3
11½ week high-F females	29.7	37.0	33.4	5.2	15.5
	31.7	31.7	31.7	0.0	0.0
	29.5	26.1	27.8	2.4	8.6
	13.4	16.4	14.9	2.1	14.2
	17.4	17.5	17.5	0.1	0.4
	24.4	26.2	25.3	1.3	5.0
	39.0	39.7	39.4	0.5	1.3
	11.6	14.5	13.1	2.1	15.7
	37.6	19.5	28.6	12.8	44.8
	25.6	36.5	31.0	7.7	24.8
	16.1	14.9	15.5	0.8	5.5
11½ week low-F males	32.9	37.8	35.4	3.5	9.8
	29.1	22.7	25.9	4.5	17.5
	27.9	35.1	31.5	5.1	16.2
	36.2	44.2	40.2	5.7	14.1
	34.2	37.0	35.6	2.0	5.6
	17.6	22.9	20.3	3.7	18.5
	54.3	56.6	55.5	1.6	2.9
	44.6	42.0	43.3	1.8	4.2
	29.2	27.7	28.5	1.1	3.7
	23.4	25.1	24.3	1.2	5.0
	28.0	28.6	28.3	0.4	1.5
11½ week high-F males	20.8	24.1	22.5	2.3	10.4
	21.5	30.3	25.9	6.2	24.0
	13.4	13.7	13.6	0.2	1.6
	9.9	12.8	11.4	2.1	18.1
	25.4	29.8	27.6	3.1	11.3
	24.6	22.3	23.5	1.6	6.9
	20.0	10.4	15.2	6.8	44.7
	26.1	33.1	29.6	4.9	16.7
	18.8	12.9	15.9	4.2	26.3
	28.1	5.9	17.0	15.7	92.3
	17.7	21.0	19.4	2.3	12.1
	21.3	25.3	23.3	2.8	12.1

Table C.8 Results of coefficients of variations between levels of aMT6s in urine collected in day 1 and day 2 by gerbils at the time of sexual maturation (on a 24-h basis).

	day 1	day 2	mean	SD	CV%
11½ week low-F females urine collected on 24-h basis	33.4	38.0	35.7	3.3	9.1
	33.9	27.1	30.5	4.8	15.6
	21.4	18.1	19.8	2.3	11.8
	15.3	**18.4**	16.9	2.2	13.1
	21.9	22.7	22.3	0.6	2.7
	32.0	30.9	31.5	0.8	2.6
	24.9	**31.5**	28.2	4.7	16.7
	26.7	28.1	27.4	1.0	3.6
	15.3	**28.3**	21.8	9.2	42.1
	21.6	21.9	21.8	0.2	0.8
	25.7	25.0	25.4	0.5	1.8
	26.8	23.9	25.4	2.0	7.9
	26.1	25.2	25.6	0.6	2.4
	25.1	24.4	24.7	0.5	2.2
	23.1	23.2	23.2	0.1	0.5
	27.5	21.4	24.5	4.3	17.7
	40.7	36.9	38.8	2.7	7.0
	12.9	11.4	12.2	1.1	8.8
11½ week high-F females urine collected on 24-h basis	28.5	29.7	29.1	0.8	2.9
	23.1	23.9	23.5	0.6	2.5
	18.9	10.9	14.9	5.7	38.0
	16.0	16.5	16.3	0.4	2.3
	19.2	**28.2**	23.7	6.3	26.7
	25.6	22.9	24.3	2.0	8.1
	15.4	20.3	17.9	3.5	19.6
	27.3	30.9	29.1	2.5	8.7
	31.1	32.9	32.0	1.2	3.9
	24.9	**34.5**	29.7	6.8	22.8
	16.6	**20.4**	18.5	2.6	14.3
	19.5	18.7	19.1	0.6	3.0
	15.9	**18.9**	17.4	2.1	12.1
11½ week high-F males urine collected on 24-h basis	15.7	14.2	15.0	1.1	7.1
	19.6	22.1	20.9	1.8	8.6
	17.8	16.8	17.3	0.7	4.3
	17.2	16.5	16.8	0.5	3.2
	14.9	**19.3**	17.1	3.1	18.0
	14.1	13.7	13.9	0.3	2.3
	23.7	16.0	19.8	5.4	27.4
	35.4	18.9	27.1	11.7	43.1
	33.3	26.6	30.0	4.7	15.7
9 week high-F males urine collected on 24-h basis	13.7	**17.9**	15.8	3.0	19.1
	19.8	**24.8**	22.3	3.6	15.9
	20.8	18.3	19.5	1.8	9.0
	31.6	24.7	28.2	4.9	17.4
	25.3	**31.6**	28.5	4.4	15.6
	24.5	23.5	24.0	0.7	3.0
	24.4	24.5	24.4	0.0	0.2
	23.9	24.1	24.0	0.2	0.8
9 week low-F males urine collected on 24-h basis	15.5	13.9	14.7	1.1	7.7
	14.5	14.7	14.6	0.2	1.1
	17.4	**21.3**	19.4	2.8	14.3
	10.0	**18.8**	14.4	6.3	43.5
	15.2	16.1	15.7	0.6	3.9
	11.2	**15.1**	13.1	2.8	21.0
	15.5	15.5	15.5	0.0	0.3

Table C.9 Results of coefficients of variations between levels of aMT6s in urine collected in day 1 and day 2 by additional gerbils at 16 weeks of age (on a 24-h basis).

	day 1	day 2	mean	SD	CV%
16 week low-F females	13.4	13.6	13.5	0.2	1.4
urine collected on a 24-h basis	12.6	14.0	13.3	1.0	7.5
	17.9	17.3	17.6	0.4	2.3
	20.6	13.1	16.9	5.3	31.6
	15.0	6.8	10.9	5.8	53.2
	16.0	13.3	14.7	1.9	13.0
16 week high-F females	19.5	16.8	18.2	1.9	10.4
urine collected on a 24-h basis	24.1	21.6	22.8	1.8	7.9
	14.0	11.9	12.9	1.5	11.7
	27.8	32.7	30.2	3.4	11.4
16 week low-F males	8.6	23.2	15.9	10.3	65.0
urine collected on a 24-h basis	21.3	22.1	21.7	0.6	2.8
	18.3	13.3	15.8	3.5	22.5
	14.5	26.2	20.4	8.3	40.8
	20.2	17.6	18.9	1.9	10.0
	28.2	16.0	22.1	8.7	39.2
	14.5	11.0	12.7	2.5	19.5
	20.9	21.0	20.9	0.1	0.4
16 week high-F males	17.1	25.0	21.1	5.6	26.5
urine collected on a 24-h basis	32.7	24.2	28.4	6.1	21.3
	23.7	28.8	26.3	3.6	13.9
	26.7	35.7	31.2	6.3	20.2
	33.7	34.7	34.2	0.8	2.2
	30.0	21.0	25.5	6.4	25.0
	24.4	18.5	21.5	4.1	19.3
	28.2	21.2	24.7	4.9	20.0

Table C.10 Results of coefficients of variations between levels of aMT6s in urine collected in day 1 and day 2 by 28-week-old gerbils

	day 1	day 2	mean	SD	CV%
28 week low-F females	18.7	29.2	23.9	7.4	30.9
	17.8	18.8	18.3	0.7	3.8
	14.9	12.4	13.7	1.7	12.7
	12.9	16.8	14.9	2.8	18.8
	14.1	8.5	11.3	4.0	35.0
	23.2	19.8	21.5	2.4	11.2
	14.8	15.4	15.1	0.4	2.8
	14.3	21.0	17.7	4.8	27.0
	7.3	11.0	9.2	2.6	28.7
	10.5	13.3	11.9	2.0	16.8
	15.8	18.8	17.3	2.1	12.1
	19.4	19.7	19.5	0.2	1.2
	22.0	12.4	17.2	6.7	39.1
	19.0	25.2	22.1	4.3	19.7
28 week high-F females	16.9	19.3	18.1	1.6	9.1
	23.8	27.5	25.7	2.6	10.2
	16.3	17.8	17.0	1.0	6.1
	17.4	16.1	16.8	0.9	5.2
	22.1	20.0	21.1	1.5	7.1
	21.2	26.0	23.6	3.3	14.2
	13.3	17.5	15.4	2.9	18.9
	13.0	24.1	18.6	7.9	42.3
	18.2	19.2	18.7	0.7	3.6
	31.5	25.6	28.5	4.1	14.4
	33.5	32.4	33.0	0.8	2.5
	15.2	19.6	17.4	3.1	18.1
	25.0	19.0	22.0	4.3	19.4
28 week low-F males	22.7	17.9	20.3	3.4	16.8
	18.8	18.3	18.5	0.3	1.7
	17.0	26.2	21.6	6.5	30.2
	13.6	24.3	18.9	7.6	40.1
	25.7	28.8	27.2	2.2	8.1
	29.6	34.1	31.9	3.2	10.1
	27.0	19.9	23.4	5.0	21.5
	20.7	23.7	22.2	2.1	9.3
	31.0	28.5	29.8	1.8	6.0
28 week high-F males	39.6	32.7	36.1	4.9	13.5
	24.4	30.2	27.3	4.1	15.1
	25.4	36.9	31.1	8.1	26.0
	28.1	26.5	27.3	1.2	4.2
	12.4	15.4	13.9	2.2	15.7
	20.7	25.7	23.2	3.5	15.3
	28.5	25.7	27.1	2.0	7.4
	27.1	38.2	32.6	7.9	24.1

Table C.11 Results of the longitudinal study on mean levels of urinary aMT6s/24-h excreted by female gerbils at 7, 9, 11½ and 16 weeks of age.

LOW - FLUORIDE FEMALES

	AGE weeks	1	2	3	4	5	6	7	8	9	10	11	12	MEAN	ST DEV	SEM
	7	37.0	21.9	21.9	36.2	37.0	28.5	27.6	25.5	27.0	20.7	17.7	20.2	26.8	6.8	2.0
mean aMT6s	9	32.9	21.2	21.6	34.4	33.2	**29.7**	27.8	27.4	22.9	22.8	22.7	12.3	25.7	6.4	1.8
ng/24-h	11½	37.9	26.2	20.2	41.7	31.7	27.0	26.3	24.8	26.1	26.3	29.5	15.3	27.8	7.1	2.0
	16	36.4	40.2	14.9	34.4	35.0	28.6	37.6	23.6	28.8	30.5	31.8	15.6	29.8	8.1	2.4
	7	876	519	489	824	849	684	611	483	572	477	386	451	602	168	49
aMT6s	9	644	386	414	599	561	**540**	523	458	439	460	452	233	476	109	31
pg/g BW/24-h	11½	597	493	351	641	450	425	468	394	489	484	549	274	468	102	29
	16	537	657	236	447	469	412	554	315	457	481	513	239	443	126	37
	7	42	42	45	44	44	42	45	53	47	43	46	45	45	3.0	0.9
body weight	9	51	55	52	58	59	55	53	60	52	50	50	53	54	3.4	1.0
g	11½	64	53	58	65	71	64	56	63	53	54	54	56	59	5.9	1.7
	16	68	61	63	77	75	69	68	75	63	64	62	66	68	5.5	1.6

HIGH - FLUORIDE FEMALES

	AGE weeks	1	2	3	4	5	6	7	8	9	10	11	12	MEAN	ST DEV	SEM
	7	27.6	15.5	25.6	14.9	12.2	14.7	14.8	15.0	14.5	27.4	17.6	17.4	18.1	5.5	1.6
mean aMT6s	9	27.3	17.6	29.9	21.0	12.0	15.9	15.7	29.6	13.2	23.8	19.5	16.7	20.2	6.2	1.8
ng/24-h	11½	33.4		31.7	27.8	14.9	17.5	25.3	39.4	13.1	**37.6**	31.0	15.5	26.1	9.5	2.7
	16	31.1		33.3	26.8	15.0	7.4	36.1	40.2	7.2	36.2	19.2	13.3	24.2	12.2	3.5
	7	510	299	482	259	264	276	263	284	279	539	425	433	359	109	31
aMT6s	9	483	311	506	348	251	301	257	552	242	444	353	332	365	106	31
pg/g BW/24-h	11½	503		471	413	280	293	336	605	218	**597**	468	289	407	133	38
	16	449		481	380	258	153	398	572	115	511	262	202	344	154	44
	7	54	52	53	58	46	53	56	53	52	51	41	40	51	5.6	1.6
body weight	9	57	57	59	61	48	53	61	54	55	54	55	50	55	4.0	1.2
g	11½	66		67	67	53	60	75	65	60	63	66	54	63	6.3	1.8
	16	69		69	70	58	49	91	70	62	71	74	66	68	10.4	3.0

N.B. High-F female No. 2 died aged 14 weeks. Data expressed as the means of the mean of 2 x 24-hour urine collections. Levels of aMT6s in day 1 were used if CV% between day 1 and day 2 > 10% (represented as shaded cells).

Table C.12 Results of the longitudinal study on the mean levels of urinary aMT6s/24-h excreted by male gerbils at 7, 9, 11½ and 16 weeks of age.

LOW -FLUORIDE MALES

	AGE weeks	1	2	3	4	5	6	7	8	9	10	11	12	MEAN	ST DEV	SEM
	7	33.6	28.0	37.9	36.5	41.5	25.4	40.2	36.9	22.2	23.3	20.0	22.6	30.7	7.9	2.3
mean aMT6s	9	24.4	25.3	36.1	36.8	30.6	26.3	31.6	42.8	21.2	18.4	19.9	21.9	27.9	7.7	2.2
ng/24-h	11½	35.4	**29.1**	31.5	40.2	35.6	20.2	55.4	43.3	28.4	24.2	24.3	28.3	33.0	9.8	2.8
	16	33.0	31.3	39.1	53.4	37.6	13.4	54.2	56.4	21.6	21.5	19.7	27.9	34.1	14.5	4.2
	7	590	561	698	662	836	474	682	695	381	417	396	432	569	148	43
aMT6s	9	348	407	549	558	507	412	420	628	333	252	319	366	425	113	33
pg/g BW/24-h	11½	454	**410**	426	574	516	297	644	570	412	288	342	456	449	112	32
	16	377	384	446	652	464	181	502	617	266	237	260	380	397	148	43
	7	57	50	54	55	50	54	59	53	58	56	51	52	54	3.0	0.9
body weight	9	70	62	66	66	60	64	75	68	64	73	62	60	66	4.9	1.4
g	11½	78	71	74	70	69	68	86	76	69	84	71	62	73	6.9	2.0
	16	88	82	88	82	81	74	108	91	81	91	76	73	85	9.6	2.8

HIGH - FLUORIDE MALES

	AGE weeks	1	2	3	4	5	6	7	8	9	10	11	12	MEAN	ST DEV	SEM
	7	12.0	28.5	13.5	14.6	19.4	14.7	15.7	16.5	14.8	16.2	16.2	14.9	16.4	4.2	1.2
mean aMT6s	9	**19.6**	30.6	13.3	15.7	20.7	15.6	19.0	19.7	15.7	17.9	23.7	23.9	19.6	4.7	1.4
ng/24-h	11½	22.5	25.9	13.6	11.4	27.6	23.4	**20.0**	29.6	**18.1**	**28.1**	19.3	23.3	21.9	5.7	1.6
	16	31.8	57.8	20.7	24.3	32.1	19.3	29.3	43.3	25.8	33.6	23.0	38.0	31.6	10.9	3.1
	7	185	496	234	258	343	275	299	292	313	338	331	335	308	76	22
aMT6s	9	**302**	476	213	284	350	245	304	293	273	297	394	410	320	75	22
pg/g BW/24-h	11½	291	344	188	173	387	333	**270**	370	**255**	**396**	250.6	326	299	74	21
	16	407	672	278	351	413	284	388	506	323	388	302	475	399	112	32
	7	65	58	58	57	57	54	53	57	47	48	49	45	54	5.8	1.7
body weight	9	65	64	62	55	59	64	63	67	58	60	60	58	61	3.5	1.0
g	11½	77	75	72	66	71	70	74	80	71	71	77	72	73	3.8	1.1
	16	78	86	75	69	78	68	76	86	80	87	76	80	78	6.1	1.8

N.B. Data expressed as the means of the mean of 2 x 24-hour urine collections. Levels of aMT6s in day 1 were used if CV% between day 1 and day 2 > 10% (represented as shaded cells).

Table C.13 Laboratory results of the levels of urinary aMT6s excreted by low-fluoride female gerbils aged 7 weeks.

5.9.92	QCs: 4, 12.7, 23.1; 3.8, 11.3, 25.3											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	67.2	86.3	89.8	99.7	90.6	78.1	77.3	88.1	71.6	90.8	105.2	83.9
aMT6s ng/ml (print out)	13.360	5.626	6.040	8.007	9.841	8.877	8.809	6.649	6.574	5.010	3.359	5.638
aMT6s ng/ml	0.53	0.23	0.24	0.32	0.39	0.36	0.35	0.27	0.26	0.20	0.13	0.23
aMT6s ng/day 1	35.9	19.4	21.7	31.9	35.7	27.7	27.2	23.4	18.8	18.2	14.1	18.9
sample necessary for error in volume of urine measurement									2.3			
aMT6s ng/day 1	35.9	19.4	21.7	31.9	35.7	27.7	27.2	23.4	21.1	18.2	14.1	18.9
DAY 2												
total volume of urine (ml)	73.7	68.7	83.2	71.5	82.5	73.5	72.4	93.0	76.2	95.4	102.3	90.3
aMT6s ng/ml (print out)	12.898	8.846	6.628	14.123	11.614	9.965	9.624	7.412	10.810	6.066	5.197	5.922
aMT6s ng/ml	0.52	0.35	0.27	0.57	0.47	0.40	0.39	0.30	0.43	0.24	0.21	0.24
aMT6s ng/day 2	38.0	24.3	22.1	40.4	38.3	29.3	27.9	27.6	32.9	23.1	21.3	21.4
aMT6s ng/48-h	73.9	43.7	43.8	72.3	74	57	55.1	51	54.1	41.3	35.4	40.3
mean aMT6s ng/24-h	37.0	21.9	21.9	36.2	37.0	28.5	27.6	25.5	27.0	20.7	17.7	20.2
body weight g	42	42	45	44	44	42	45	53	47	43	46	45
mean aMT6s pg/g BW/24-h	876	519	489	824	849	684	611	483	572	477	386	451

Table C.14 Laboratory results of the levels of urinary aMT6s excreted by high-fluoride female gerbils aged 7 weeks.

29.7.92	QCs: 4, 12.7, 23.1; 3.8, 11.3, 25.3											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	55.1	49.0	45.3	47.1	67.9	55.4	88.0	66.0	61.1	48.7	62.9	62.6
aMT6s ng/ml (print out)	12.933	6.153	14.609	8.041	4.142	5.788	3.265	5.5	5.4	12.5	6.9	7.258
aMT6s ng/ml	0.52	0.25	0.58	0.32	0.17	0.23	0.13	0.22	0.22	0.50	0.28	0.29
aMT6s ng/day 1	28.5	12.1	26.5	15.1	11.2	12.8	11.5	14.4	13.1	24.4	17.3	18.2
DAY 2												
total volume of urine (ml)	89.6	74.1	96.8	56.8	100.4	72.9	86.1	97.5	79.6	73.6	79.9	85.4
aMT6s ng/ml (print out)	7.427	6.4	6.379	6.449	3.28	5.678	5.274	4.0	5.0	10.4	5.6	4.856
aMT6s ng/ml	0.30	0.26	0.26	0.26	0.13	0.23	0.21	0.16	0.20	0.41	0.22	0.19
aMT6s ng/day 2	26.6	19.0	24.7	14.7	13.2	16.6	18.2	15.7	15.9	30.5	17.8	16.6
aMT6s ng/48-h	55.1	31.0	51.2	29.8	24.4	29.4	29.7	30.1	29.1	54.9	35.1	34.8
mean aMT6s ng/24-h	27.6	15.5	25.6	14.9	12.2	14.7	14.8	15.0	14.5	27.4	17.6	17.4
body weight g	54	52	53	58	46	53	56	53	52	51	41	40
mean aMT6s pg/g BW/24-h	510	299	482	259	264	276	263	284	279	539	425	433

Table C.15 Laboratory results of the levels of urinary aMT6s excreted by low-fluoride male gerbils aged 7 weeks.

5.9.92	QCs: 3.6, 14.2, 24.1; 3.3, 14.7,26.4											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	50.0	69.3	82.6	76.8	75.9	90.3	73.7	72.8	102.4	97.0	85.5	94.9
aMT6s ng/ml (print out)	15.632	9.746	10.957	11.419	12.777	6.657	13.672	9.8605	5.474	4.966	6.143	4.899
aMT6s ng/ml	0.63	0.39	0.44	0.46	0.51	0.27	0.55	0.39	0.22	0.20	0.25	0.20
aMT6s ng/day 1	31.3	27.0	36.2	38.6	38.8	24.0	40.3	28.7	22.4	19.3	21.0	18.6
DAY 2												
total volume of urine (ml)	71.3	82.1	95.2	85.8	89.4	108.5	100.7	81.0	98.7	105.2	87.9	91.4
aMT6s ng/ml (print out)	12.588	lost	10.393	9.0335	12.343	6.153	9.951	13.924	5.573	6.484	5.3925	7.273
aMT6s ng/ml	0.50	done by circadian data	0.42	0.40	0.49	0.25	0.40	0.56	0.22	0.26	0.22	0.29
aMT6s ng/day 2	35.9	29.0	39.6	34.3	44.1	26.7	40.1	45.1	22.0	27.3	19.0	26.6
aMT6s ng/48-h	67.2	56.0	75.8	72.9	82.9	50.7	80.4	73.8	44.4	46.6	40.0	45.2
mean aMT6s ng/24-h	33.6	28.0	37.9	36.5	41.5	25.4	40.2	36.9	22.2	23.3	20.0	22.6
body weight g	57	50	54	55	50	54	59	53	58	56	51	52
mean aMT6s pg/g BW/24-h	590	561	698	662	836	474	682	695	381	417	396	432

Table C.16 Laboratory results of the levels of urinary aMT6s excreted by high-fluoride male gerbils aged 7 weeks.

29.7.92	QCs: 3.6, 14.2, 24.1; 3.3, 14.7, 26.4											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	56.6	54.8	58.3	51.4	45.7	65.4	48.8	57.7	63.2	73.8	67.2	85.9
aMT6s ng/ml (print out)	3.944	11.434	4.886	5.813	3.771	5.098	8.108	6.8	5.932	4.817	5.552	3.693
aMT6s ng/ml	0.16	0.46	0.20	0.23	0.15	0.20	0.32	0.27	0.24	0.19	0.22	0.15
aMT6s ng/day 1	8.9	25.1	11.4	12.0	6.9	13.3	15.8	15.7	15.0	14.2	14.9	12.7
DAY 2												
total volume of urine (ml)	96.1	79.1	84.2	70.0	63.3	81.0	60.7	94.8	94.9	81.1	93.7	95.1
aMT6s ng/ml (print out)	3.938	10.123	4.631	6.19	12.596	4.966	6.442	4.576	3.859	5.607	4.649	4.504
aMT6s ng/ml	0.16	0.40	0.19	0.25	0.50	0.20	0.26	0.18	0.15	0.22	0.19	0.18
aMT6s ng/day 2	15.1	32.0	15.6	17.3	31.9	16.1	15.6	17.4	14.6	18.2	17.4	17.1
aMT6s ng/48-h	24.1	57.1	27.0	29.3	38.8	29.4	31.5	33.0	29.6	32.4	32.3	29.8
mean aMT6s ng/24-h	12.0	28.5	13.5	14.6	19.4	14.7	15.7	16.5	14.8	16.2	16.2	14.9
body weight g	65	58	58	57	57	54	53	57	47	48	49	45
mean aMT6s pg/g BW/24-h	185	496	234	258	343	275	299	292	313	338	331	335

Table C.17 Laboratory results of the levels of urinary aMT6s excreted by low-fluoride female gerbils aged 9 weeks. (Levels in day 1 for No 6 taken as the mean aMT6s/24-h).

21.9.92	**QCs: 3.9, 13.8, 20.3; 4.1, 12.7, 20.6**											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	66.1	141.8	93.8	97.2	104.6	90.6	84.4	84.9	90.4	95.3	94.9	84.3
aMT6s ng/ml (print out)	11.099	3.456	6.030	8.877	7.963	8.200	8.274	6.686	6.527	6.020	5.326	3.718
aMT6s ng/ml	0.44	0.14	0.24	0.36	0.32	0.33	0.33	0.27	0.26	0.24	0.21	0.15
aMT6s ng/day 1	29.3	19.6	22.6	34.5	33.3	**29.7**	27.9	22.7	23.6	22.9	20.2	12.5
DAY 2												
total volume of urine (ml)	82	106.6	85.2	110.4	99.3	107.9	103.2	88.2	lost	88.9	102.6	106.6
aMT6s ng/ml (print out)	11.114	5.367	6.010	7.776	8.343	5.968	6.704	9.102	used	6.379	6.160	2.813
aMT6s ng/ml	0.44	0.21	0.24	0.31	0.33	0.24	0.27	0.36	circadian data	0.26	0.25	0.11
aMT6s ng/day 2	36.5	22.9	20.5	34.3	33.1	25.8	27.7	32.1	22.1	22.7	25.3	12.0
aMT6s ng/48-h	65.8	42.5	43.1	68.9	66.5	55.5	55.6	54.8	45.7	45.6	45.5	24.5
mean aMT6s ng/24-h	32.9	21.2	21.6	34.4	33.2	**29.7**	27.8	27.4	22.9	22.8	22.7	12.3
body weight g	51	55	52	58	59	55	53	60	52	50	50	53
mean aMT6s pg/g BW/24-h	644	386	414	599	561	**538**	523	458	439	460	452	233

Table C.18 Laboratory results of the levels of urinary aMT6s excreted by high-fluoride female gerbils aged 9 weeks.

12.8.92	QCs: 3.9, 13.8, 20.3; 4.1, 12.7, 20.6											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	72.2	70.4	76.0	68.7	75.2	81.2	58.3	63.6	78.8	76.7	65.7	73.0
aMT6s ng/ml (print out)	9.604	4.631	7.637	6.248	3.970	4.013	5.629	8.355	4.389	5.788	6.241	5.104
aMT6s ng/ml	0.38	0.19	0.31	0.25	0.16	0.16	0.23	0.33	0.18	0.23	0.25	0.20
aMT6s ng/day 1	27.7	13.0	23.2	17.2	11.9	13.0	13.1	21.3	13.8	17.8	16.4	14.9
DAY 2												
total volume of urine (ml)	85.1	79.1	84.2	79.5	97.7	75.7	76.9	84.3	107.1	121.3	76.1	82.1
aMT6s ng/ml (print out)	7.913	6.066	10.861	7.836	3.101	5.350	5.958	11.258	2.944	6.146	7.432	4.553
aMT6s ng/ml	0.32	0.24	0.43	0.31	0.12	0.21	0.24	0.45	0.12	0.25	0.30	0.18
aMT6s ng/day 2	26.9	19.2	36.6	24.9	12.1	16.2	18.3	38.0	12.6	29.8	22.6	15.0
errors in urine vol collection		2.9				2.5						3.5
aMT6s ng/day 2	26.9	22.1	36.6	24.9	12.1	18.7	18.3	38.0	12.6	29.8	22.6	18.5
aMT6s ng/48-h	54.7	35.2	59.8	42.1	24.1	31.7	31.5	59.2	26.4	47.6	39.0	33.4
mean aMT6s ng/24-h	27.3	17.6	29.9	21.0	12.0	15.9	15.7	29.6	13.2	23.8	19.5	16.7
body weight g	57	57	59	61	48	53	61	54	55	54	55	50
mean aMT6s pg/g BW/24-h	483	311	506	348	251	301	257	552	242	444	353	332

Table C.19 Laboratory results of the levels of urinary aMT6s excreted by low-fluoride male gerbils aged 9 weeks.

21.9.92	QCs: 3.6, 14.5, 25.9; 3.7, 11.8, 22.8											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	77.0	100.8	89.5	93.1	94.3	82.8	74.9	92.0	104.9	83.6	88.5	97.9
aMT6s ng/ml (print out)	6.901	5.932	9.544	9.064	7.500	8.159	9.766	11.473	4.438	5.205	4.707	5.200
aMT6s ng/ml	0.28	0.24	0.38	0.36	0.30	0.33	0.39	0.46	0.18	0.21	0.19	0.21
aMT6s ng/day 1	21.3	23.9	34.2	33.8	28.3	27.0	29.3	42.2	18.6	17.4	16.7	20.4
DAY 2												
total volume of urine (ml)	89.6	101.8	97.8	95.2	87.5	96.7	95.4	114.0	105.0	105.6	92.0	122.1
aMT6s ng/ml (print out)	7.701	6.534	9.739	10.464	8.257	6.599	8.902	9.498	5.681	4.571	6.268	4.809
aMT6s ng/ml	0.31	0.26	0.39	0.42	0.33	0.26	0.36	0.38	0.23	0.18	0.25	0.19
aMT6s ng/day 2	27.6	26.6	38.1	39.8	32.9	25.5	34.0	43.3	23.9	19.3	23.1	23.5
including extra due to error in urine measurement	4.0											
aMT6s ng / 48 h	48.9	50.5	72.3	73.6	61.2	52.5	63.2	85.5	42.5	36.7	39.7	43.9
mean aMT6s ng / 24h	24.4	25.3	36.1	36.8	30.6	26.3	31.6	42.8	21.2	18.4	19.9	21.9
body weight g	70	62	66	66	60	64	75	68	64	73	62	60
mean aMT6s pg / g b wt / 24 h	348	407	549	558	507	412	420	628	333	252	319	366

Table C.20 Laboratory results of the levels of urinary aMT6s excreted by high-fluoride male gerbils aged 9 weeks. (Shaded cells represent where the levels of aMT6s in day 1 are taken as the mean aMT6s/24-h).

12.8.92	**QCs: 3.6, 14.5, 25.9; 3.7, 11.8, 22.8**											
DAY 1	**No 1**	**No 2**	**No 3**	**No 4**	**No 5**	**No 6**	**No 7**	**No 8**	**No 9**	**No 10**	**No 11**	**No 12**
total volume of urine (ml)	58.3	53.7	58.1	57.8	65.8	47.4	65.9	71.5	73.2	74.8	88.0	78.3
aMT6s ng/ml (print out)	8.407	9.425	4.902	5.186	5.977	8.002	6.879	5.791	4.689	5.062	6.375	6.901
aMT6s ng/ml	0.34	0.38	0.20	0.21	0.24	0.32	0.28	0.23	0.19	0.20	0.26	0.28
aMT6s ng/day 1	**19.6**	20.2	11.4	12.0	15.7	15.2	18.1	16.6	13.7	15.1	22.4	21.6
DAY 2												
total volume of urine (ml)	80.0	57.7	67.0	81.6	79.8	57.8	95.1	81.4	78.0	83.1	75.9	89.3
aMT6s ng/ml (print out)	4.170	17.778	5.656	5.919	8.035	6.943	5.240	7.039	5.675	6.190	8.194	7.340
aMT6s ng/ml	0.17	0.71	0.23	0.24	0.32	0.28	0.21	0.28	0.23	0.25	0.33	0.29
aMT6s ng/day 2	13.3	41.0	15.2	19.3	25.6	16.1	19.9	22.9	17.7	20.6	24.9	26.2
aMT6s ng/48-h	32.9	61.3	26.6	31.3	41.4	31.2	38.1	39.5	31.4	35.7	47.3	47.8
mean aMT6s ng/24-h	**19.6**	30.6	13.3	15.7	20.7	15.6	19.0	19.7	15.7	17.9	23.7	23.9
body weight g	65	64	62	55	59	64	63	67	58	60	60	58
mean aMT6s pg/g BW/24-h	**303**	476	213	284	350	245	304	293	273	297	394	410

Table C.21(i) Laboratory results of circadian excretion of urinary aMT6s over 48-h from low-F female gerbils aged 11½ weeks.

QCs: 3.6, 14.2, 22.3

7.10.92	11½ W LF F1			11½ W LF F2			11½ W LF F3			11½ W LF F4		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1300 - 1600	11.9	0.3040	3.6	8.5	0.0282	0.2	6.9	0.1439	1.0	9.1	0.2430	2.2
1600 - 1900	10.5	0.2033	2.1	11.8	0.1759	2.1	11.7	0.2661	3.1	9.0	0.3450	3.1
1900 - 2200	10.0	0.2369	2.4	9.6	0.0377	0.4	7.8	0.2723	2.1	9.8	0.2390	2.3
2200 - 0100	14.7	0.1130	1.7	11.8	0.2238	2.6	10.0	0.1300	1.3	13.0	0.4460	5.8
0100 - 0400	15.4	1.0964	16.9	13.7	0.6701	9.2	13.8	0.1079	1.5	12.7	1.0030	12.7
0400 - 0700	8.9	0.0448	0.4	9.1	0.1691	1.5	12.0	0.7474	9.0	10.8	1.0680	11.5
0700 - 1000	10.5	0.7029	7.4	19.0	0.2994	5.7	14.1	0.0631	0.9	7.0	0.4150	2.9
1000 - 1300	4.9	0.0280	0.1	9.1	0.0827	0.8	12.4	0.1360	1.7	12.5	0.1510	1.9
1300 - 1600	11.8	0.4641	5.5	13.5	0.3199	4.7	10.4	0.1562	1.6	14.5	0.1530	2.2
1600 - 1900	14.0	0.2930	4.1	16.4	0.0288	0.5	11.5	0.1850	2.1	15.9	0.2880	4.6
1900 - 2200	15.5	0.2262	3.5	18.2	0.2259	4.1	13.7	0.1320	1.8	14.4	0.1110	1.6
2200 - 0100	13.2	0.2650	3.5	13.4	0.3312	4.4	14.0	0.1810	2.5	12.2	0.5900	7.2
0100 - 0400	16.0	0.7029	11.2	11.8	0.0346	0.4	8.7	0.4540	3.9	12.1	0.8800	10.6
0400 - 0700	10.7	0.5981	6.4	14.2	0.5043	7.2	12.4	0.2540	3.1	10.4	0.7270	7.6
0700 - 1000	9.1	0.4174	3.8	12.5	0.4611	5.8	9.4	0.1660	1.6	8.7	0.2840	2.5
1000 - 1300	8.3	0.3846	3.2	9.6	0.2926	2.8	10.1	0.3120	3.2	17.7	0.2610	4.6
total aMT6s ng/48-h			75.8			52.3			40.5			83.4
total aMT6s ng/day 1			34.6			22.5			20.6			42.5
total aMT6s ng/day 2			41.2			29.9			19.9			40.9
mean aMT6s ng/24-h			37.9			26.2			20.2			41.7
body weight g			64			53			58			65
aMT6s pg/g BW/24-h			597			494			349			642

Table C.21(ii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from low-F female gerbils aged 11½ weeks.

QCs: 3.5, 13.1, 19.8

7.10.92	11½ W LF F5			11½ W LF F6			11½W LF F7			11½ W LF F8		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	10.4	0.2524	2.6	20.0	0.1369	2.7	8.1	0.1105	0.9	0.0	0.0010	0.0
1600 - 1900	14.4	0.0947	1.4	0.0	0.0011	0.0	9.3	0.0572	0.5	10.8	0.0916	1.0
1900 - 2200	11.6	0.0869	1.0	17.0	0.0442	0.8	12.5	0.0830	1.0	12.1	0.1161	1.4
2200 - 0100	9.3	0.3027	2.8	12.4	0.0445	0.6	13.0	0.0308	0.4	10.8	0.0011	0.0
0100 - 0400	10.0	0.5948	5.9	11.0	0.3619	4.0	9.4	1.2402	11.7	7.5	0.8332	6.2
0400 - 0700	8.4	1.0664	9.0	11.6	1.0082	11.7	11.0	0.7793	8.6	11.3	1.0722	12.1
0700 - 1000	8.9	0.6852	6.1	9.9	0.3746	3.7	8.3	0.0145	0.1	16.0	0.1780	2.8
1000 - 1300	10.4	0.1408	1.5	19.4	0.0426	0.8	9.2	0.0067	0.1	9.3	0.0030	0.0
1300 - 1600	12.0	0.0975	1.2	13.5	0.0348	0.5	11.5	0.1641	1.9	6.5	0.3864	2.5
1600 - 1900	12.6	0.4573	5.8	10.0	0.1238	1.2	11.6	0.0852	1.0	6.4	0.0092	0.1
1900 - 2200	16.6	0.1526	2.5	16.8	0.2169	3.6	11.8	0.1587	1.9	14.8	0.0127	0.2
2200 - 0100	10.7	0.1278	1.4	11.4	0.4418	5.0	7.5	0.3451	2.6	14.9	0.1245	1.9
0100 - 0400	20.7	0.1901	3.9	15.0	0.6939	10.4	13.7	0.6534	9.0	16.0	0.6538	10.5
0400 - 0700	14.9	0.8718	13.0	13.9	0.3292	4.6	15.0	0.6382	9.6	16.7	0.4366	7.3
0700 - 1000	13.2	0.2828	3.7	22.0	0.1846	4.1	11.8	0.0881	1.0	14.5	0.1060	1.5
1000 - 1300	11.7	0.1340	1.6	9.1	0.2559	0.3	13.2	0.1630	2.2	12.2	0.1716	2.1
total aMT6s ng/48-h			63.4			54.0			52.5			49.6
total aMT6s ng/day 1			30.3			24.3			23.3			23.5
total aMT6s ng/day 2			33.1			29.7			29.2			26.1
mean aMT6s ng/24-h			31.7			27.0			26.3			24.8
body weight g			71			64			56			63
aMT6s pg/g BW/24-h			450			422			469			394

Table C.21(iii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from low-F female gerbils aged 11½ weeks.

QCs: 3.6, 13.3, 21.2

7.10.92	11½ W LF F9			11½ W LF F10			12WLF F11			11½ W LF F12		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1300 - 1600	5.3	0.1287	0.7	1.6	0.0278	0.0	2.6	0.0071	0.0	5.8	0.1170	0.7
1600 - 1900	12.3	0.1355	1.7	12.0	0.2742	3.3	12.2	0.1582	1.9	9.5	0.2457	2.3
1900 - 2200	11.7	0.1698	2.0	12.2	0.1600	2.0	11.2	0.1600	1.8	11.0	0.1036	1.1
2200 - 0100	13.7	0.1895	2.6	12.8	0.0281	0.4	14.6	0.2196	3.2	16.1	0.1661	2.7
0100 - 0400	11.1	0.7133	7.9	9.2	0.0265	0.2	11.6	0.7755	9.0	10.5	0.1550	1.6
0400 - 0700	9.5	0.8211	7.8	14.5	0.7324	10.6	13.0	0.1084	1.4	6.2	0.4225	2.6
0700 - 1000	16.2	0.0239	0.4	14.5	0.4044	5.9	13.5	0.0328	0.4	12.7	0.2372	3.0
1000 - 1300	9.5	0.3022	2.9	7.6	0.0601	0.5	13.5	0.7952	10.7	8.9	0.1163	1.0
1300 - 1600	17.4	0.1339	2.3	14.7	0.3012	4.4	18.5	0.1976	3.7	10.2	0.2140	2.2
1600 - 1900	8.4	0.0259	0.2	9.2	0.3024	2.8	7.5	0.1999	1.5	12.0	0.0916	1.1
1900 - 2200	16.5	0.1958	3.2	15.7	0.1716	2.7	13.0	0.1356	1.8	16.5	0.1120	1.8
2200 - 0100	16.8	0.0194	0.3	13.6	0.0292	0.4	13.9	0.1381	1.9	19.2	0.1016	2.0
0100 - 0400	12.9	0.3787	4.9	13.9	0.3276	4.6	10.4	0.5062	5.3	11.6	0.1589	1.8
0400 - 0700	11.5	0.6200	7.1	14.3	0.7432	10.6	14.6	0.3721	5.4	12.0	0.3230	3.9
0700 - 1000	18.0	0.3398	6.1	9.6	0.3447	3.3	10.7	0.8142	8.7	17.5	0.1079	1.9
1000 - 1300	9.9	0.2080	2.1	7.7	0.1117	0.9	13.6	0.1663	2.3	12.9	0.0656	0.8
total aMT6s ng/48-h			52.2			52.6			59.0			30.5
total aMT6s ng/day 1			26.0			22.9			28.4			15.0
total aMT6s ng/day 2			26.2			29.7			30.6			15.5
mean aMT6s ng/24-h			26.1			26.3			29.5			15.3
body weight g			53			54			54			56
aMT6s pg/g BW/24-h			489			487			546			272

Table C.22 (i) Laboratory results of circadian excretion of urinary aMT6s over 48-h from high-F female gerbils aged 11½ weeks.

QCs: 3.7, 12.5, 21

30.8.92	11½ W HF F1			11½ W HF F2			11½ W HF F3			11½ W HF F4		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	9.5	0.0851	0.8	8.9	0.0962	0.9	6.2	0.0289	0.2	8.2	0.1384	1.1
1600 - 1900	8.0	0.4337	3.5	7.6	0.1015	0.8	9.2	0.1218	1.1	13.5	0.1667	2.3
1900 - 2200	4.4	0.4328	1.9	7.6	0.1394	1.1	17.8	0.0776	1.4	12.2	0.1603	2.0
2200 - 0100	9.2	0.2404	2.2	10.0	0.0805	0.8	8.0	0.7064	5.7	11.2	0.1953	2.2
0100 - 0400	4.9	1.4550	7.1	9.8	0.0916	0.9	7.6	1.6094	12.2	9.4	1.0744	10.1
0400 - 0700	10.3	0.6804	7.0	10.8	0.0913	1.0	8.6	0.8120	7.0	10.8	0.6671	7.2
0700 - 1000	18.1	0.1932	3.5	13.1	0.0971	1.3	12.2	0.2734	3.3	14.1	0.1824	2.6
1000 - 1300	9.4	0.3914	3.7	7.0	0.1167	0.8	10.2	0.0790	0.8	26.5	0.0805	2.1
1300 - 1600	8.5	0.4275	3.6	9.8	0.0618	0.6	11.5	0.2634	3.0	11.6	0.1376	1.6
1600 - 1900	13.5	0.2524	3.4	9.5	0.0735	0.7	11.6	0.0919	1.1	8.9	0.3393	3.0
1900 - 2200	8.5	0.3458	2.9	10.7	0.0886	0.9	8.9	0.0993	0.9	6.6	0.3352	2.2
2200 - 0100	9.3	0.3316	3.1	12.8	0.0521	0.7	7.2	0.1681	1.2	10.1	0.2924	3.0
0100 - 0400	8.2	0.8148	6.7	9.7	0.1264	1.2	4.6	1.7233	7.9	10.3	0.4318	4.4
0400 - 0700	8.6	1.0274	8.8	12.7	0.1180	1.5	9.3	1.6738	15.6	10.3	0.4762	4.9
0700 - 1000	16.0	0.2063	3.3	13.2	0.1344	1.8	9.8	0.1580	1.5	11.5	0.4057	4.7
1000 - 1300	9.8	0.5254	5.1	7.6	0.1479	1.1	4.4	0.1120	0.5	7.8	0.2916	2.3
total aMT6s ng/48-h			66.7			16.0			63.4			55.6
total aMT6s ng/day 1			29.7			7.5			31.7			29.5
total aMT6s ng/day 2			37.0			8.5			31.7			26.1
mean aMT6s ng/24-h			33.4			8.0			31.7			27.8
body weight g			66			52			67			67
aMT6s pg/g BW/24-h			503			154			471			413

Table C.22 (ii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from high-F female gerbils aged 11½ weeks.

QCs: 3.4, 12.7, 26.0												
30.8.92	**11½ W HF F5**			**11½ W HF F6**			**11½ W HF F7**			**11½ W HF F8**		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	7.9	0.0840	0.7	7.7	0.1232	0.9	8.3	0.2027	1.7	4.8	0.0652	0.3
1600 - 1900	8.4	0.2108	1.8	8.3	0.1939	1.6	11.4	0.0927	1.1	10.0	0.2633	2.6
1900 - 2200	17.2	0.0467	0.8	19.2	0.1012	1.9	11.6	0.2353	2.7	18.5	0.0847	1.6
2200 - 0100	8.9	0.3723	3.3	13.9	0.2014	2.8	9.8	0.0407	0.4	11.1	0.8194	9.1
0100 - 0400	9.8	0.2606	2.6	9.6	0.4147	4.0	9.5	1.0288	9.8	10.0	1.4889	14.9
0400 - 0700	7.0	0.2440	1.7	16.5	0.1712	2.8	23.8	0.1874	4.5	6.6	0.9000	5.9
0700 - 1000	11.7	0.1027	1.2	9.9	0.1780	1.8	11.4	0.1194	1.4	11.0	0.2027	2.2
1000 - 1300	25.0	0.0565	1.4	16.7	0.0921	1.5	15.0	0.1953	2.9	5.8	0.4044	2.3
1300 - 1600	8.0	0.0437	0.3	10.0	0.1047	1.0	9.9	0.2395	2.4	9.8	0.0675	0.7
1600 - 1900	21.3	0.1089	2.3	10.5	0.1644	1.7	8.4	0.4220	3.5	7.2	0.7168	5.2
1900 - 2200	8.1	0.1288	1.0	10.9	0.1587	1.7	11.5	0.1454	1.7	13.7	0.2430	3.3
2200 - 0100	13.0	0.2278	3.0	9.9	0.1092	1.1	10.5	0.3039	3.2	14.9	0.1965	2.9
0100 - 0400	8.3	0.3508	2.9	12.8	0.2950	3.8	12.2	0.5205	6.4	10.2	7.9460	16.2
0400 - 0700	12.3	0.3508	4.3	7.7	0.5356	4.1	11.0	0.2350	2.6	4.9	8.5250	8.4
0700 - 1000	13.6	0.1108	1.5	14.7	0.1744	2.6	19.9	0.1926	3.8	10.5	0.2299	2.4
1000 - 1300	8.5	0.1180	1.0	9.2	0.1605	1.5	13.6	0.1931	2.6	12.4	0.0519	0.6
total aMT6s ng/48-h			29.8			34.9			50.6			78.7
total aMT6s ng/day 1			13.4			17.4			24.4			39.0
total aMT6s ng/day 2			16.4			17.5			26.2			39.7
mean aMT6s ng/24-h			14.9			17.5			25.3			39.4
body weight g			53			60			75			65
aMT6s pg/g BW/24-h			280			293			336			605

Table C.22 (iii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from high-F female gerbils aged 11½ weeks. (Shaded cells represent where the levels of aMT6s in day 1 are taken as mean aMT6s/24-h).

QCs: 3.7, 14.1, 22.3

30.8.92	11½ W HF F9			11½ W HF F10			11½ W HF F11			11½ W HF F12		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	9.7	0.1109	1.1	6.3	0.3373	2.1	10.4	0.1481	1.5	10.0	0.1089	1.1
1600 - 1900	12.5	0.1570	2.0	9.6	0.1973	1.9	10.2	0.2259	2.3	7.1	0.2178	1.5
1900 - 2200	12.2	0.1553	1.9	15.5	0.1110	1.7	8.9	0.1515	1.3	16.0	0.0335	0.5
2200 - 0100	11.4	0.1572	1.8	9.2	1.1598	10.7	13.2	0.2399	3.2	8.0	0.2740	2.2
0100 - 0400	7.0	0.1989	1.4	10.1	1.3056	13.2	12.8	0.0338	0.4	10.2	0.1432	1.5
0400 - 0700	12.2	0.1297	1.6	13.4	0.3272	4.4	11.5	1.1326	13.0	13.0	0.4586	6.0
0700 - 1000	12.3	0.0157	0.2	12.8	0.2497	3.2	12.2	0.0752	0.9	14.4	0.2003	2.9
1000 - 1300	8.9	0.1873	1.7	7.7	0.0537	0.4	15.8	0.1814	2.9	5.9	0.0799	0.5
1300 - 1600	12.7	0.1188	1.5	14.7	0.2104	3.1	13.9	0.2608	3.6	16.8	0.1316	2.2
1600 - 1900	17.1	0.1507	2.6	11.1	0.3513	3.9	10.6	0.2729	2.9	10.4	0.3880	4.0
1900 - 2200	11.3	0.1247	1.4	11.8	0.0407	0.5	10.5	0.2573	2.7	10.2	0.0424	0.4
2200 - 0100	22.1	0.1024	2.3	11.5	0.2952	3.4	8.9	0.5426	4.8	11.6	0.0364	0.4
0100 - 0400	10.1	0.1802	1.8	12.7	0.2033	2.6	13.7	0.6928	9.5	10.8	0.0422	0.5
0400 - 0700	11.0	0.1742	1.9	6.8	0.2339	1.6	9.8	0.6424	6.3	8.7	0.3221	2.8
0700 - 1000	8.3	0.1636	1.4	12.4	0.3350	4.2	17.9	0.1365	2.4	11.7	0.1266	1.5
1000 - 1300	8.3	0.2026	1.7	8.2	0.0424	0.3	9.2	0.4563	4.2	11.5	0.2674	3.1
total aMT6s ng/48-h			26.1			57.1			62.1			31.1
total aMT6s ng/day 1			11.6			37.6			25.6			16.1
total aMT6s ng/day 2			14.5			19.5			36.5			14.9
mean aMT6s ng/24-h			13.0			37.6			31.0			15.5
body weight g			60			63			66			54
aMT6s pg/g BW/24-h			218			595			468			289

Table C.23 (i) Laboratory results of circadian excretion of urinary aMT6s over 48-h from low-F male gerbils aged 11½ weeks. Shaded cells represent where the levels of aMT6s in day 1 are taken as mean aMT6s/24-h.

QCs: 3.5, 13.4, 22.1

7.10.92	11½ W LF M1			11½ W LF M 2			11½ W LF M3			11½W LF M4		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	0.1	0.0010	0.0	9.3	0.1451	1.3	0.8	0.0012	0.0	8.2	0.2830	2.3
1600 - 1900	7.9	0.4220	3.3	9.4	0.5191	4.9	7.5	0.3743	2.8	6.2	0.0010	0.0
1900 - 2200	11.5	0.2464	2.8	8.4	0.4337	3.6	12.8	0.0008	0.0	17.2	0.1080	1.9
2200 - 0100	14.8	0.2578	3.8	12.0	0.3014	3.6	12.0	0.3221	3.9	17.7	0.2230	3.9
0100 - 0400	12.2	0.0063	0.1	12.1	0.4770	5.8	10.5	0.0009	0.0	11.5	0.0010	0.0
0400 - 0700	9.0	1.5728	14.2	9.5	0.5964	5.7	9.9	1.6750	16.6	11.7	2.3940	28.0
0700 - 1000	13.4	0.6456	8.7	12.6	0.0541	0.7	13.5	0.0037	0.0	9.6	0.0020	0.0
1000 - 1300	7.8	0.0082	0.1	12.5	0.2802	3.5	9.9	0.4601	4.6	9.4	0.0010	0.0
1300 - 1600	12.2	0.5169	6.3	8.8	0.2633	2.3	10.4	0.0023	0.0	9.2	0.6170	5.7
1600 - 1900	12.0	0.2055	2.5	10.8	0.1399	1.5	13.6	0.5240	7.1	10.4	0.3290	3.4
1900 - 2200	11.1	0.2600	2.9	12.7	0.1981	2.5	15.0	0.1421	2.1	14.7	0.0010	0.0
2200 - 0100	13.2	0.1108	1.5	11.0	0.1070	1.2	11.5	0.3310	3.8	14.5	0.2380	3.5
0100 - 0400	10.5	0.3658	3.8	8.6	0.6577	5.7	12.9	0.0020	0.0	17.0	0.8170	13.9
0400 - 0700	15.0	1.0686	16.0	18.8	0.2745	5.2	15.5	1.0600	16.4	12.5	0.8210	10.3
0700 - 1000	14.6	0.1161	1.7	18.6	0.1783	3.3	25.5	0.1600	4.1	16.2	0.3000	4.9
1000 - 1300	14.8	0.2103	3.1	14.9	0.0640	1.0	10.8	0.1380	1.5	15.0	0.1780	2.7
total aMT6s ng/48-h			70.7			51.8			63.0			80.4
total aMT6s ng/day 1			32.9			29.1			27.9			36.2
total aMT6s ng/day 2			37.8			22.7			35.1			44.2
mean aMT6s ng/24-h			35.4			29.1			31.5			40.2
body weight g			78			71			74			70
aMT6s pg/g BW/24-h			456			410			427			572

Table C.23 (ii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from low-F male gerbils aged 11½ weeks.

QCs: 3.7, 14.7, 24.7

7.10.92	11½ W LF M5			11½ W LF M6			11½ W LF M7			11½W LF M8		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	2.7	0.0024	0.0	2.2	0.0023	0.0	9.2	0.5038	4.6	7.2	0.3343	2.4
1600 - 1900	12.1	0.2518	3.0	13.9	0.1021	1.4	10.5	0.1267	1.3	7.8	0.0832	0.6
1900 - 2200	9.6	0.2643	2.5	14.5	0.0463	0.7	17.0	0.1054	1.8	15.7	0.1011	1.6
2200 - 0100	16.3	0.2271	3.7	14.1	0.0661	0.9	14.4	0.7005	10.1	15.0	0.1664	2.5
0100 - 0400	10.0	1.0599	10.6	14.9	0.3976	5.9	11.0	1.5256	16.8	10.8	1.2461	13.5
0400 - 0700	9.6	1.1653	11.2	14.3	0.4271	6.1	3.2	0.0032	0.0	11.4	0.9192	10.5
0700 - 1000	11.5	0.1651	1.9	6.4	0.3849	2.5	14.6	0.9944	14.5	14.1	0.7766	11.0
1000 - 1300	8.4	0.1507	1.3	13.0	0.0038	0.0	12.0	0.4285	5.1	15.8	0.1646	2.6
1300 - 1600	12.4	0.1976	2.5	10.7	0.2482	2.7	7.7	0.0036	0.0	12.0	0.1771	2.1
1600 - 1900	8.8	0.1949	1.7	19.0	0.0472	0.9	11.2	0.7128	8.0	11.2	0.2518	2.8
1900 - 2200	15.7	0.1151	1.8	15.5	0.0324	0.5	15.8	0.1839	2.9	15.5	0.2140	3.3
2200 - 0100	13.5	0.4994	6.7	15.6	0.0885	1.4	13.2	0.1775	2.3	12.0	0.2395	2.9
0100 - 0400	13.8	0.6723	9.3	13.7	0.2813	3.9	15.0	0.8413	12.6	14.4	1.1748	16.9
0400 - 0700	11.1	0.7463	8.3	13.2	0.8194	10.8	13.6	1.4383	19.6	10.6	0.8274	8.8
0700 - 1000	8.4	0.6096	5.1	12.0	0.1499	1.8	14.0	0.5528	7.7	9.5	0.3059	2.9
1000 - 1300	15.0	0.1049	1.6	12.7	0.0769	1.0	9.3	0.3641	3.4	13.7	0.1623	2.2
total aMT6s ng/48-h			71.2			40.5			110.9			86.6
total aMT6s ng/day 1			34.2			17.6			54.3			44.6
total aMT6s ng/day 2			37.0			22.9			56.6			42.0
mean aMT6s ng/24-h			35.6			20.2			55.4			43.3
body weight g			69			68			86			76
aMT6s pg/g BW/24-h			516			299			647			570

Table C.23 (iii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from low-F male gerbils aged 11½ weeks.

QCs: 3.3, 12.4, 24.9

7.10.92	11½ W LF M9			11½ W LF M10			11½ W LF M11			11½W LF M12		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	3.0	0.0895	0.3	0.0	0.0010	0.0	8.1	0.2430	2.0	0.0	0.0090	0.0
1600 - 1900	15.2	0.2191	3.3	9.5	0.1415	1.3	9.9	0.2659	2.6	12.1	0.2747	3.3
1900 - 2200	13.5	0.1715	2.3	14.8	0.2232	3.3	12.0	0.2450	2.9	14.7	0.1044	1.5
2200 - 0100	13.1	0.2940	3.9	14.2	0.2650	3.8	10.3	0.1965	2.0	12.6	0.2474	3.1
0100 - 0400	14.3	0.1235	1.8	14.9	0.4192	6.2	11.8	0.0893	1.1	20.5	0.1069	2.2
0400 - 0700	10.5	1.0861	11.4	12.2	0.3731	4.6	8.4	0.6017	5.1	10.2	1.0839	11.1
0700 - 1000	13.7	0.3449	4.7	5.2	0.1004	0.5	13.4	0.3386	4.5	12.8	0.4232	5.4
1000 - 1300	10.6	0.1433	1.5	16.0	0.3039	4.9	14.3	0.2245	3.2	12.4	0.1094	1.4
1300 - 1600	11.2	0.2284	2.6	10.1	0.2998	3.0	12.7	0.2241	2.8	13.5	0.2649	3.6
1600 - 1900	12.4	0.2480	3.1	10.4	0.1574	1.6	9.8	0.1608	1.6	11.1	0.2832	3.1
1900 - 2200	17.0	0.1741	3.0	12.9	0.2583	3.3	16.1	0.2094	3.4	0.0	0.0918	0.0
2200 - 0100	11.2	0.2259	2.5	12.7	0.0001	0.0	13.0	0.2224	2.9	12.5	0.2223	2.8
0100 - 0400	13.5	0.4073	5.5	12.7	0.3670	4.7	10.5	0.3118	3.3	11.4	0.6599	7.5
0400 - 0700	11.7	0.4651	5.4	15.3	0.2450	3.7	11.2	0.2859	3.2	17.1	0.4269	7.3
0700 - 1000	8.4	0.3426	2.9	12.1	0.3169	3.8	14.4	0.4188	6.0	12.7	0.2573	3.3
1000 - 1300	10.1	0.2705	2.7	21.6	0.1623	3.5	9.5	0.1995	1.9	9.4	0.1127	1.1
total aMT6s ng/48-h			56.9			48.3			48.5			56.6
total aMT6s ng/day 1			29.2			24.6			23.4			28.0
total aMT6s ng/day 2			27.7			23.7			25.1			28.6
mean aMT6s ng/24-h			28.4			24.2			24.3			28.3
body weight g			69			84			71			62
aMT6s pg/g BW/24-h			412			289			342			458

Table C.24 (i) Laboratory results of circadian excretion of urinary aMT6s over 48-h from high-F male gerbils aged 11½ weeks.

QCs: 3.9, 14.1, 26.1

30.8.92	11½ W HF M1			11½ W HF M2			11½ W HF M3			11½ W HF M4		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1300 - 1600	3.7	0.0049	0.0	1.8	0.6344	1.1	7.3	0.0997	0.7	7.2	0.0698	0.5
1600 - 1900	3.6	0.9102	3.3	11.0	0.0669	0.7	7.5	0.2610	2.0	8.1	0.0633	0.5
1900 - 2200	7.7	0.2597	2.0	6.0	0.0049	0.0	5.3	0.1989	1.1	7.4	0.1829	1.4
2200 - 0100	8.9	0.3318	3.0	5.7	0.1617	0.9	5.2	0.1110	0.6	6.2	0.2470	1.5
0100 - 0400	6.1	0.4252	2.6	7.7	8.6030	13.2	7.0	0.4770	3.3	6.4	0.1300	0.8
0400 - 0700	4.8	0.0093	0.0	4.3	0.2930	1.3	7.2	0.1432	1.0	6.9	0.2633	1.8
0700 - 1000	9.3	0.7078	6.6	12.5	0.1563	2.0	15.6	0.1900	3.0	13.9	0.1093	1.5
1000 - 1300	9.6	0.3513	3.4	6.3	0.0282	0.2	10.5	0.1677	1.8	9.7	0.0631	0.6
1300 - 1600	6.2	0.0170	0.1	11.1	0.0064	0.1	8.9	0.0135	0.1	8.6	0.2654	2.3
1600 - 1900	9.8	0.2298	2.3	9.5	0.5974	5.7	10.2	0.1244	1.3	9.0	0.1320	1.2
1900 - 2200	11.0	0.2169	2.4	10.5	0.2097	2.2	9.2	0.1820	1.7	10.5	0.1033	1.1
2200 - 0100	11.2	0.0069	0.1	12.4	0.5098	6.3	4.0	0.2008	0.8	10.8	0.0915	1.0
0100 - 0400	12.5	0.9551	11.9	14.2	0.0155	0.2	10.7	0.3570	3.8	8.6	0.3624	3.1
0400 - 0700	6.1	0.0076	0.0	10.4	1.2794	13.3	7.4	0.5029	3.7	9.7	0.2828	2.7
0700 - 1000	6.8	1.0068	6.8	5.6	0.4138	2.3	26.6	0.0371	1.0	10.0	0.0824	0.8
1000 - 1300	16.0	0.0277	0.4	11.0	0.2016	2.2	20.5	0.0640	1.3	12.6	0.1460	1.8
total aMT6s ng/48-h			44.9			51.8			27.1			22.7
total aMT6s ng/day 1			20.8			19.5			13.4			8.7
total aMT6s ng/day 2			24.1			32.3			13.7			14.1
mean aMT6s ng/24-h			22.5			25.9			13.6			11.4
body weight g			77			75			72			66
aMT6s pg/g BW/24-h			291			344			188			173

Table C.24 (ii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from high-F male gerbils aged 11½ weeks. Shaded cells represent where the levels of aMT6s in day 1 are taken as mean aMT6s/24-h.

QCs: 3.7, 13.2, 22.5

30.8.92	11½ W HF M5			11½ W HF M6			11½ W HF M7			11½ W HF M8		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	8.8	0.2142	1.9	7.5	0.0718	0.5	5.7	0.0055	0.0	2.2	0.0029	0.0
1600 - 1900	7.4	0.2346	1.7	7.5	0.4775	3.6	12.4	0.1367	1.7	9.0	0.2137	1.9
1900 - 2200	9.9	0.3091	3.1	9.1	0.0049	0.0	15.0	0.0993	1.5	10.8	0.1973	2.1
2200 - 0100	6.4	0.4682	3.0	7.0	0.2588	1.8	9.9	0.3148	3.1	11.6	0.3419	4.0
0100 - 0400	4.3	1.0379	4.5	8.7	0.6977	6.1	5.6	1.1804	6.6	9.0	0.9875	8.9
0400 - 0700	13.5	0.0314	0.4	6.6	0.0034	0.0	8.0	0.4309	3.4	9.9	0.5459	5.4
0700 - 1000	10.6	0.6642	7.0	12.0	0.9432	11.3	12.5	0.0073	0.1	6.4	0.0221	0.1
1000 - 1300	11.4	0.3327	3.8	5.0	0.0077	0.0	10.5	0.3343	3.5	15.8	0.2305	3.6
1300 - 1600	6.8	0.0354	0.2	5.6	0.4073	2.3	12.2	0.1396	1.7	12.5	0.2490	3.1
1600 - 1900	11.5	0.2245	2.6	9.7	0.1907	1.8	8.1	0.2760	2.2	9.5	0.4583	4.4
1900 - 2200	8.7	0.2753	2.4	7.1	0.2449	1.7	9.5	0.0045	0.0	6.0	0.4283	2.6
2200 - 0100	10.0	0.4636	4.6	6.1	0.2599	1.6	8.7	0.3208	2.8	6.2	0.0044	0.0
0100 - 0400	12.5	0.3698	4.6	5.9	0.2896	1.7	3.6	0.0076	0.0	12.7	0.3815	4.8
0400 - 0700	9.3	0.8724	8.1	11.0	0.8615	9.5	3.4	0.1637	0.6	9.4	1.0393	9.8
0700 - 1000	16.0	0.1691	2.7	14.6	0.2720	4.0	14.7	0.1262	1.9	15.0	0.4115	6.2
1000 - 1300	18.1	0.2509	4.5	20.6	0.0396	0.8	10.5	0.1111	1.2	16.3	0.1362	2.2
total aMT6s ng/48-h			55.2			46.9			30.4			59.2
total aMT6s ng/day 1			25.4			23.4			20.0			26.1
total aMT6s ng/day 2			29.8			23.4			10.4			33.1
mean aMT6s ng/24-h			27.6			23.4			**20.0**			29.6
body weight g			71			70			74			80
aMT6s pg/g BW/24-h			387			333			**271**			372

Table C.24 (iii) Laboratory results of circadian excretion of urinary aMT6s over 48-h from high-F male gerbils aged 11½ weeks. Shaded cells represent where the levels of aMT6s in day 1 are taken as mean aMT6s/24-h.

QCs: 3.5, 13.2, 19												
30.8.92	**11½ W HF M9**			**11½ W HF M10**			**11½ W HF M11**			**11½ W HF M12**		
TIME	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**	**volume ml**	**aMT6s ng/ml**	**aMT6s ng**
1300 - 1600	12.0	0.0437	0.5	7.7	0.0002	0.0	11.1	0.0466	0.5	8.4	0.0028	0.0
1600 - 1900	7.2	0.0002	0.0	9.2	0.2670	2.5	14.6	0.0372	0.5	15.2	0.1327	2.0
1900 - 2200	10.0	0.3698	3.7	10.5	0.1858	2.0	11.8	0.2670	3.2	10.1	0.2731	2.8
2200 - 0100	13.0	0.0003	0.0	9.6	0.0039	0.0	14.3	0.1639	2.3	15.8	0.2926	4.6
0100 - 0400	8.7	0.9986	8.7	13.8	0.9224	12.7	13.5	0.2590	3.5	11.5	0.2978	3.4
0400 - 0700	8.0	0.5558	4.4	8.2	0.0002	0.0	6.6	0.7776	5.1	4.2	0.0007	0.0
0700 - 1000	15.5	0.0002	0.0	9.6	0.6992	6.7	11.5	0.1191	1.4	14.7	0.4754	7.0
1000 - 1300	18.3	0.0387	0.7	8.4	0.5007	4.2	11.4	0.1034	1.2	4.6	0.0085	0.0
1300 - 1600	11.4	0.1341	1.5	7.2	0.0006	0.0	10.0	0.0715	0.7	17.2	0.0096	0.2
1600 - 1900	14.7	0.1046	1.5	8.1	0.2411	2.0	12.8	0.0437	0.6	14.7	0.2823	4.1
1900 - 2200	11.6	0.2284	2.6	10.5	0.2120	2.2	5.8	0.5747	3.3	8.3	0.1710	1.4
2200 - 0100	12.1	0.0158	0.2	11.1	0.0003	0.0	8.0	0.1814	1.5	11.7	0.5010	5.9
0100 - 0400	10.3	0.2731	2.8	11.0	0.1340	1.5	8.5	0.6924	5.9	11.6	0.4375	5.1
0400 - 0700	8.0	0.3043	2.4	15.6	0.0090	0.1	10.0	0.4102	4.1	18.1	0.3373	6.1
0700 - 1000	12.5	0.0973	1.2	13.6	0.0068	0.1	16.0	0.1401	2.2	12.0	0.0101	0.1
1000 - 1300	10.9	0.1157	1.3	14.7	0.0002	0.0	13.7	0.1947	2.7	17.0	0.2258	3.8
total aMT6s ng/48-h			31.7			34.0			38.7			46.6
total aMT6s ng/day 1			18.1			28.1			17.7			19.9
total aMT6s ng/day 2			13.6			5.9			21.0			26.7
mean aMT6s ng/24-h			18.1			28.1			19.3			23.3
body weight g			71			71			77			72
aMT6s pg/g BW/24-h			255			396			253			326

Table C.25 Laboratory results of circadian excretion of urinary aMT6s over 24-h from low-F female gerbils aged 16 weeks on 8.11.92.

QCs: 3.5, 14.3, 19.5	16WLF F1			16WLF F2			16WLF F3			16WLF F4		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1800 - 2100	14.6	1.315	1.9	8.2	6.875	5.6	10.8	0.760	0.8	12.9	1.248	1.6
2100 - 2400	7.5	6.036	4.5	7.9	6.819	5.4	8.5	2.259	1.9	13.4	1.965	2.6
2400 - 0300	13.9	0.315	0.4	11.3	0.316	0.4	9.5	2.581	2.5	9.3	0.285	0.3
0300 - 0600	9.6	9.972^	19.1	9.6	12.881	12.4	12.7	3.084	3.9	13.0	0.314	0.4
0600 - 0900	6.9	0.314	0.2	8.8	10.088	8.9	15.3	1.157	1.8	16.9	12.708	21.5
0900 - 1200	8.9	5.663	5.0	8.2	3.912	3.2	6.1	0.355	0.2	6.8	0.295	0.2
1200 - 1500	11.1	0.285	0.3	11.3	0.428	0.5	9.2	3.841	3.5	12.0	3.708	4.4
1500 - 1800	9.4	5.135	4.8	8.2	4.764	3.9	6.3	0.352	0.2	14.4	2.359	3.4
total aMT6s ng/24-h			36.4			40.2			14.9			34.4
body weight g			68			61			63			77
aMT6s pg/g BW/24-h			537			657			236			447
QCs: 3.5, 14.3, 19.5	16WLF F5			16WLF F6			16WLF F7			16WLF F8		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1800 - 2100	12.6	1.136	1.4	13.4	2.552	3.4	14.4	1.369	2.0	10.9	0.333	0.4
2100 - 2400	8.7	1.739	1.5	10.2	2.022	2.1	9.5	2.692	2.6	7.3	2.922	2.1
2400 - 0300	9.5	4.071	3.9	10.2	5.312	5.4	10.4	7.798	8.1	13.2	7.173	9.5
0300 - 0600	10.5	13.506	14.2	10.1	6.382	7.1	9.6	15.910	15.3	12.4	3.989	4.9
0600 - 0900	9.6	3.370	3.2	11.3	2.218	2.5	11.4	2.861	3.3	6.3	0.599	0.4
0900 - 1200	9.3	4.325	4.0	8.3	3.812	3.2	9.8	2.209	2.2	12.9	1.800	2.3
1200 - 1500	13.0	1.472	1.9	9.8	1.053	1.0	14.5	1.123	1.6	16.2	2.312	3.7
1500 - 1800	7.9	6.146	4.9	11.1	3.513	3.9	8.6	3.016	2.6	5.4	0.375	0.2
total aMT6s ng/24-h			35.0			28.6			37.6			23.6
body weight g			75			69			68			75
aMT6s pg/g BW/24-h			469			412			554			315
QCs: 3.8, 14.9, 25.4	16WLF F9			16WLF F10			16WLF F11			16WLF F12		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1800 - 2100	18.1	0.664	1.2	9.8	3.470	3.4	13.5	1.566	2.1	13.0	3.350	4.4
2100 - 2400	6.7	1.734	1.2	7.9	1.849	1.5	9.7	0.259	0.3	5.8	1.521	0.9
2400 - 0300	17.1	4.556	7.8	11.4	3.359	3.8	11.4	4.318	4.9	19.4	0.727	1.4
0300 - 0600	6.5	13.000	8.5	3.6	0.294	0.1	12.3	12.811	15.8	10.5	2.892	3.0
0600 - 0900	2.7	0.221	0.1	8.3	14.798	12.3	2.5	0.583	0.1	12.8	1.380	1.8
0900 - 1200	12.9	3.764	4.9	12.6	4.088	5.2	20.0	2.604	5.2	14.6	0.741	1.1
1200 - 1500	11.6	2.354	2.7	12.6	0.316	0.4	6.8	0.320	0.2	6.3	3.733	2.4
1500 - 1800	14.7	1.701	2.5	17.4	2.241	3.9	11.9	2.698	3.2	9.5	0.782	0.7
total aMT6s ng/24-h			28.8			30.5			31.8			15.6
body weight g			63			64			62			66
aMT6s pg/g BW/24-h			457			481			513			239

Table C.26 Laboratory results of circadian excretion of urinary aMT6s over 24-h from high-F female gerbils aged 16 weeks on 1.10.92.

QCs: 3.6, 13.2, 23.1	16WHF F1			16WHF F2			16WHF F3			16WHF F4		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1600 - 1900	6.6	0.347	2.3	10.0	0.254	2.5	9.8	0.288	2.8	12.5	0.022	0.3
1900 - 2200	6.8	0.295	2.0	11.5	0.022	0.3	10.0	0.261	2.6	9.1	0.413	3.8
2200 - 0100	11.9	0.037	0.4	14.5	0.182	2.6	13.7	0.140	1.9	16.7	0.024	0.4
0100 - 0400	9.2	1.133	10.4	11.5	0.021	0.2	10.8	0.676	7.3	16.5	0.357	5.9
0400 - 0700	14.4	0.759	10.9	11.3	0.948	10.7	19.7	0.664	13.1	14.2	0.845	12.0
0700 - 1000	8.4	0.036	0.3	11.8	0.317	3.7	11.7	0.037	0.4	8.2	0.026	0.2
1000 - 1300	10.1	0.453	4.6	12.5	0.028	0.3	10.6	0.332	3.5	10.5	0.383	4.0
1300 - 1600	7.9	0.024	0.2	7.9	0.488	3.9	11.6	0.143	1.7	7.6	0.025	0.2
total aMT6s ng/24-h			31.1			24.3			33.3			26.8
body weight g			69			66			69			70
aMT6s pg/g BW/24-h			449			371			481			380
QCs: 3.8, 13.7, 22.5	16WHF F5			16WHF F6			16WHF F7			16WHF F8		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1600 - 1900	11.2	0.133	1.5	8.5	0.037	0.3	7.8	0.316	2.5	13.8	0.090	1.2
1900 - 2200	10.5	0.070	0.7	12.2	0.052	0.6	10.5	0.009	0.1	12.2	0.138	1.7
2200 - 0100	11.5	0.128	1.5	16.4	0.017	0.3	8.2	1.215	10.0	14.5	0.011	0.2
0100 - 0400	12.8	0.072	0.9	16.6	0.071	1.2	12.1	0.103	1.2	11.9	7.448^	17.7
0400 - 0700	16.1	0.372	6.0	18.9	0.088	1.7	9.5	0.010	0.1	11.8	0.014	0.2
0700 - 1000	10.9	0.118	1.3	9.8	0.140	1.4	10.2	7.908^	16.1	9.2	8.46^	15.6
1000 - 1300	4.5	0.430	1.9	13.2	0.056	0.7	8.3	0.727	6.0	17.5	0.013	0.2
1300 - 1600	9.4	0.130	1.2	8.8	0.140	1.2	5.2	0.017	0.1	6.0	0.573	3.4
total aMT6s ng/24-h			15.0			7.4			36.1			40.2
body weight g			58			49			91			70
aMT6s pg/g BW/24-h			258			153			398			572
QCs: 3.6, 12.6, 22.1	16WHF F9			16WHF F10			16WHF F11			16WHF F12		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1600 - 1900	11.8	0.030	0.4	12.1	0.101	1.2	11.8	0.079	0.9	9.8	0.004	0.0
1900 - 2200	10.8	0.046	0.5	21.0	0.041	0.9	14.2	0.060	0.9	20.5	0.033	0.7
2200 - 0100	12.1	0.029	0.4	10.0	0.579	5.8	14.5	0.162	2.4	12.4	0.007	0.1
0100 - 0400	13.7	0.034	0.5	10.5	1.259	13.2	19.1	0.333	6.4	17.3	0.200	3.5
0400 - 0700	18.3	0.080	1.5	9.2	0.986	9.1	10.1	0.478	4.8	7.8	0.004	0.0
0700 - 1000	11.5	0.157	1.8	7.3	0.399	2.9	5.3	0.221	1.2	17.5	0.392	6.9
1000 - 1300	8.4	0.025	0.2	8.1	0.004	0.0	17.7	0.149	2.6	9.6	0.225	2.2
1300 - 1600	8.4	0.239	2.0	9.2	0.331	3.0	6.7	0.014	0.1	4.4	0.004	0.0
total aMT6s ng/24-h			7.2			36.2			19.2			13.3
body weight g			62			71			74			66
aMT6s pg/g BW/24-h			115			511			262			202

Table C.27 Laboratory results of circadian excretion of urinary aMT6s over 24-h from low-F male gerbils aged 16 weeks on 8.11.92.

QCs: 3.9, 15.3, 25.5	16WLF M1			16WLF M2			16WLF M3			16WLF M4		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1800 - 2100	9.4	0.311	0.3	12.5	2.025	2.5	13.2	1.096	1.4	12.5	0.351	0.4
2100 - 2400	12.7	2.296	2.9	13.8	3.126	4.3	9.3	4.430	4.1	12.2	9.739	11.9
2400 - 0300	17.8	4.780	8.5	10.1	0.408	0.4	13.6	11.574	15.7	11.5	0.348	0.4
0300 - 0600	8.7	11.669	10.2	12.6	11.076	14.0	8.0	7.831	6.3	11.8	14.970	17.7
0600 - 0900	6.8	4.354	3.0	8.4	3.165	2.7	8.5	4.202	3.6	10.5	12.452	13.1
0900 - 1200	10.0	0.449	0.4	9.4	2.877	2.7	5.3	0.392	0.2	12.1	0.816	1.0
1200 - 1500	7.0	5.788	4.1	13 6	1.716	2.3	9.8	5.283	5.2	12.7	2.770	3.5
1500 - 1800	12.4	2.950	3.7	12.1	1.987	2.4	15.8	1.613	2.5	9.6	5.616	5.4
total aMT6s ng/24-h			33.0			31.3			39.1			53.4
body weight g			88			82			88			82
aMT6s pg/g BW/24-h			377			384			446			652
QCs: 4.0, 14.1, 23.2	16WLF M5			16WLF M6			16WLF M7			16WLF M8		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1800 - 2100	19.3	0.285	0.6	15.0	0.878	1.3	15.3	1.913	2.9	13.7	1.706	2.3
2100 - 2400	11.4	4.113	4.7	10.5	1.021	1.1	11.2	4.285	4.8	11.3	3.594	4.1
2400 - 0300	9.2	8.125	7.5	11.2	1.980	2.2	11.7	9.256	10.8	13.0	9.944	12.9
0300 - 0600	9.7	14.075	13.7	5.9	4.586	2.7	7.9	22.447	17.7	10.6	22.010	23.3
0600 - 0900	7.6	5.065	3.8	11.5	1.474	1.7	10.4	5.607	5.8	8.1	4.443	3.6
0900 - 1200	11.0	1.425	1.6	12.4	1.001	1.2	12.9	0.530	0.7	12.7	2.110	2.7
1200 - 1500	9.9	2.602	2.6	13.6	1.467	2.0	11.2	4.889	5.5	10.5	2.548	2.7
1500 - 1800	11.0	2.964	3.3	11.2	1.065	1.2	8.3	7.074	5.9	16.6	2.884	4.8
total aMT6s ng/24-h			37.6			13.4			54.2			56.4
body weight g			81			74			108			91
aMT6s pg/g BW/24-h			464			181			502			617
QCs: 3.7, 14.1, 26.5	16WLF M9			16WLF M10			16WLF M11			16WLF M12		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1800 - 2100	15.2	2.252	3.4	18.2	1.435	2.6	10.1	3.272	3.3	16.6	1.984	3.3
2100 - 2400	10.5	0.337	0.4	15.7	1.541	2.4	14.5	1.597	2.3	14.2	0.308	0.4
2400 - 0300	14.7	0.315	0.5	12.6	3.448	4.3	11.2	1.317	1.5	12.4	6.327	7.8
0300 - 0600	10.3	6.886	7.1	12.8	3.893	5.0	9.6	4.209	4.0	11.4	0.293	0.3
0600 - 0900	10.2	3.465	3.5	10.4	2.191	2.3	8.9	2.918	2.6	9.8	7.935	7.8
0900 - 1200	10.8	2.073	2.2	11.2	0.244	0.3	13.8	1.279	1.8	9.1	0.574	0.5
1200 - 1500	18.0	0.963	1.7	10.0	2.723	2.7	12.2	2.571	3.1	15.3	3.110	4.8
1500 - 1800	15.1	1.830	2.8	11.5	1.606	1.8	11.4	0.973	1.1	13.6	2.168	2.9
total aMT6s ng/24-h			21.6			21.5			19.7			27.9
body weight g			81			91			76			73
aMT6s pg/g BW/24-h			266			237			260			380

Table C.28 Laboratory results of circadian excretion of urinary aMT6s over 24-h from high-F male gerbils aged 16 weeks on 1.10.92.

QCs: 3.8, 14.9, 25.4	16WHF M1			16WHF M2			16WHF M3			16WHF M4		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1600 - 1900	10.6	0.260	0.3	9.8	2.450	2.4	6.4	2.500	1.6	11.0	1.481	1.6
1900 - 2200	9.7	4.566	4.4	7.6	5.320	4.0	16.8	0.755	1.3	11.6	1.461	1.7
2200 - 0100	11.4	0.308	0.4	9.0	0.708	0.6	9.6	2.123	2.0	13.4	1.465	2.0
0100 - 0400	12.1	0.284	0.3	14.5	14.708	21.3	12.5	3.078	3.8	8.5	3.710	3.2
0400 - 0700	14.1	13.379	18.9	6.7	5.183	3.5	10.9	4.361	4.8	15.1	6.679	10.1
0700 - 1000	8.4	0.368	0.3	13.1	14.009	18.4	8.6	2.894	2.5	18.5	1.154	2.1
1000 - 1300	8.4	0.382	0.3	10.2	0.512	0.5	9.9	2.629	2.6	10.0	1.808	1.8
1300 - 1600	10.2	6.819	7.0	11.3	6.248	7.1	11.0	1.915	2.1	8.8	2.110	1.9
total aMT6s ng/24-h			31.8			57.8			20.7			24.3
body weight g			78			86			75			69
aMT6s pg/g BW/24-h			407			671			278			351
QC's: 3.7, 14.5, 25.3	16WHF M5			16WHF M6			16WHF M7			16WHF M8		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1600 - 1900	11.7	2.374	2.8	14.7	2.187	3.2	16.3	1.502	2.4	8.4	3.068	2.6
1900 - 2200	12.0	3.339	4.0	12.3	0.354	0.4	13.8	0.288	0.4	13.4	2.883	3.9
2200 - 0100	11.7	0.237	0.3	10.7	4.283	4.6	13.0	2.492	3.2	9.6	0.348	0.3
0100 - 0400	12.2	4.276	5.2	11.1	0.319	0.4	8.9	12.402	11.0	10.2	9.910	10.1
0400 - 0700	10.8	12.419	13.4	15.4	3.546	5.5	8.7	6.649	5.8	9.9	15.102	15.0
0700 - 1000	9.9	2.406	2.4	10.4	2.601	2.7	13.8	0.519	0.7	8.3	3.648	6.1
1000 - 1300	10.6	3.520	3.7	15.8	1.378	2.2	13.6	2.200	3.0	9.4	0.459	0.4
1300 - 1600	8.9	0.371	0.3	6.3	0.510	0.3	8.9	3.031	2.7	8.3	6.027	5.0
total aMT6s ng/24-h			32.1			19.3			29.3			43.3
body weight g			78			68			76			86
aMT6s pg/g BW/24-h			413			284			388			506
QCs: 3.9, 14.9, 21.1	16WHF M9			16WHF M10			16WHF M11			16WHF M12		
TIME	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng	volume ml	aMT6s ng/ml	aMT6s ng
1600 - 1900	13.5	0.379	0.5	12.5	4.215	5.3	11.0	0.059	0.1	17.6	0.751	1.3
1900 - 2200	15.0	2.732	4.1	12.0	3.146	3.8	12.4	2.678	3.3	14.6	0.048	0.1
2200 - 0100	8.8	0.390	0.3	9.1	0.040	0.0	12.9	2.003	2.6	13.6	4.751	6.5
0100 - 0400	10.0	4.397	4.4	14.8	9.121	13.5	9.6	0.041	0.0	10.1	0.051	0.1
0400 - 0700	12.4	9.834	12.2	12.2	0.050	0.1	13.4	8.706	11.7	14.5	14.047	20.4
0700 - 1000	7.6	0.551	0.4	13.2	4.994	6.6	12.4	1.276	1.6	10.6	9.058	9.6
1000 - 1300	11.6	2.962	3.4	6.1	0.042	0.0	4.8	0.065	0.0	8.8	0.111	0.1
1300 - 1600	9.9	0.409	0.4	8.9	4.872	4.3	12.1	3.099	3.7	9.6	0.047	0.0
total aMT6s ng/24-h			25.8			33.6			23.0			38.0
body weight g			80			87			76			80
aMT6s pg/g BW/24-h			323			388			302			475

Table C.29 Laboratory results of urinary levels of aMT6s in female gerbils aged 11½ weeks over 48 hours in 2 x 24-hour intervals. Shaded cells indicate where day 1 levels of aMT6s were used in statistical analyses.

11½ week high-fluoride female gerbils

	DAY 1			DAY 2						
QCs: 3.3, 13.0, 24.3	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
11½W HF F1	11.303	12.6	28.5	10.521	14.1	29.7	58.2	29.1	76	384
11½W HF F2	9.617	12.0	23.1	7.974	15.0	23.9	47.0	23.5	63	375
11½W HF F3	9.551	9.9	**18.9**	6.491	8.4	10.9	29.8	**18.9**	67	281
11½W HF F4	5.332	15.0	16.0	5.619	14.7	16.5	32.5	16.3	71	228
11½W HF F5	5.492	17.5	19.2	11.828	11.9	28.2	47.4	23.7	70	337
11½W HF F6	7.771	16.5	25.6	4.553	25.1	22.9	48.5	24.3	68	355
11½W HF F7	7.334	10.5	15.4	6.204	16.4	20.3	35.8	17.9	61	295
11½W HF F8	7.386	18.5	27.3	7.355	21.0	30.9	58.2	29.1	58	498
11½W HF F9	6.403	24.3	31.1	7.755	21.2	32.9	64.0	32.0	55	580
11½W HF F10	6.595	18.9	24.9	9.746	17.7	34.5	59.4	29.7	63	475
11½W HF F11	5.616	14.8	16.6	5.549	16.7*	20.4	37.0	18.5	51	362
11½W HF F12	5.922	16.5	19.5	5.385	17.4	18.7	38.3	19.1	48	397
11½W HF F13	4.494	17.7	15.9	6.89	13.7	18.9	34.8	17.4	57	303

11½ week low-fluoride female gerbils

	DAY 1			DAY 2						
QCs: 3.8, 12.9, 25.3	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
11½WLF F1	11.582	14.4	33.4	11.716	16.2	38.0	71.3	35.7	56	633
11½WLF F2	8.188	20.7	**33.9**	6.255	21.7	27.1	61.0	**33.9**	56	607
11½WLF F3	5.788	18.5	**21.4**	5.845	15.5	18.1	39.5	**21.4**	57	373
11½WLF F4	4.156	18.4	15.3	4.749	19.4	18.4	33.7	16.9	60	281
11½WLF F5	4.966	22.0	21.9	6.069	18.7	22.7	44.5	22.3	56	400
11½WLF F6	8.091	19.8	32.0	11.884	13.0	30.9	62.9	31.5	54	578
11½WLF F7	9.958	12.5	24.9	10.514	15.0	31.5	56.4	28.2	55	509
11½WLF F8	8.343	16.0	26.7	10.485	13.4	28.1	54.8	27.4	65	423
11½WLF F9	5.397	14.2	15.3	8.234	17.2	28.3	43.7	21.8	61	356
11½WLF F10	8.651	12.5	21.6	5.043	21.7	21.9	43.5	21.8	58	376
11½WLF F11	8.615	14.9	25.7	8.693	14.4	25.0	50.7	25.4	66	383
11½WLF F12	12.402	10.8	26.8	12.469	9.6	23.9	50.7	25.4	63	406
11½WLF F13	7.946	16.4	26.1	5.781	21.8	25.2	51.3	25.6	65	394
11½WLF F14	7.526	16.7	25.1	7.858	15.5	24.4	49.5	24.7	59	417
11½WLF F15	10.407	10.1*	23.1	7.088	16.4	23.2	46.3	23.2	58	397
11½WLF F16	6.879	20.0	**27.5**	4.953	21.6	21.4	48.9	**27.5**	65	426
11½WLF F17	17.706	11.5	40.7	10.657	17.3	36.9	77.6	38.8	54	725
11½WLF F18	4.276	15.1	12.9	3.066	18.6	11.4	24.3	12.2	59	205

Table C.30 Laboratory results of urinary excretion of aMT6s in pubertal male gerbils over 48 hours in 2 x 24-hour intervals. Shaded cells indicate where day 1 levels of aMT6s were used in statistical analyses.

PUBERTAL HIGH-FLUORIDE MALES

QCs: 3.8, 12.9, 25.3	DAY 1			DAY 2						
9 week high-F males	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 1**	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 2**	**aMT6s ng/48-h**	**mean aMT6s ng/24-h**	**body wt g**	**aMT6s pg/g BW/24-h**
9W HF M1	7.198	9.5	13.7	5.126	17.5	17.9	31.6	15.8	62	255
9W HF M2	4.309	23.0	19.8	7.913	15.7	24.8	44.7	22.3	65	346
9W HF M3	7.108	14.6	20.8	3.936	23.2	18.3	39.0	19.5	53	371
9W HF M4	17.001	9.3	**31.6**	5.367	23.0	24.7	56.3	**31.6**	64	491
9W HF M5	7.278	17.4	25.3	7.319	21.6	31.6	56.9	28.5	55	521
9W HF M6	6.073	20.2	24.5	8.402	14.0	23.5	48.1	24.0	56	426
9W HF M7	6.782	18.0	24.4	6.275	19.5	24.5	48.9	24.4	68	360
9W HF M8	5.188	23.0	23.9	9.432	12.8	24.1	48.0	24.0	70	345
11½ week high-F males	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 1**	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 2**	**aMT6s ng/48-h**	**mean aMT6s ng/24-h**	**body wt g**	**aMT6s pg/g BW/24-h**
11½W HF M1	3.83	20.5	15.7	2.764	25.7	14.2	29.9	15.0	62	242
11½W HF M2	5.24	18.7	19.6	6.187	17.9	22.1	41.7	20.9	68	306
11½W HF M3	6.499	13.7	17.8	4.929	17.0	16.8	34.6	17.3	62	279
11½W HF M4	8.615	10.0	17.2	3.594	22.9	16.5	33.7	16.8	59	284
11½W HF M5	4.361	17.1	14.9	3.851	25.0	19.3	34.2	17.1	62	275
11½W HF M6	4.283	16.5	14.1	4.751	14.4	13.7	27.8	13.9	68	204
11½W HF M7	7.401	16.0	**23.7**	6.344	12.6	16.0	39.7	**23.7**	67	352
11½W HF M8	10.412	17.0	**35.4**	7.258	13.0	18.9	54.3	**35.4**	69	515
11½W HF M9	9.192	18.1	**33.3**	9.185	14.5	26.6	59.9	**33.3**	71	467

PUBERTAL LOW-FLUORIDE MALES

QCs: 3.8, 12.9, 25.3	DAY 1			DAY 2						
9 week low-F males	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 1**	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 2**	**aMT6s ng/48-h**	**mean aMT6s ng/24-h**	**body wt g**	**aMT6s pg/g BW/24-h**
9W LF M1	6.449	12.0	15.5	4.447	15.6	13.9	29.4	14.7	67	219
9W LF M2	3.391	21.4	14.5	4.062	16.5 *	14.7	29.3	14.6	62	237
9W LF M3	3.997	21.8	17.4	4.621	23.1	21.3	38.8	19.4	63	309
9W LF M4	2.966	16.8	10.0	3.841	24.5	18.8	28.8	14.4	61	238
9W LF M5	3.546	21.5	15.2	4.687	17.2	16.1	31.4	15.7	58	271
9W LF M6	2.511	22.3	11.2	3.683	20.5	15.1	26.3	13.1	54	244
9W LF M7	3.87	20.0	15.5	3.517	22.1	15.5	31.0	15.5	67	230

Table C.31 Laboratory results of urinary levels of aMT6s excreted by 16-week-old gerbils over 48-hours in 2 x 24-hour intervals. Shaded cells indicate where the day 1 levels of aMT6s were used in statistical analyses.

16 week low-F females

QCs: 3.8, 13.1, 24.7	DAY 1			DAY 2						
	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
16W LF F1	3.756	17.8	13.4	3.789	18.0	13.6	27.0	13.5	71	189
16W LF F2	2.072	30.3	12.6	3.089	22.6	14.0	26.5	13.3	60	221
16W LF F3	5.528	16.2	17.9	4.290	20.2	17.3	35.2	17.6	67	265
16W LF F4	6.534	15.8	**20.6**	2.299	28.5	13.1	33.8	**20.6**	68	305
16W LF F5	4.501	16.7	**15.0**	2.271	15.0	6.8	21.8	**15.0**	53	286
16W LF F6	4.661	17.2	**16.0**	2.723	24.5	13.3	29.4	**16.0**	61	263

16 week high-fluoride females

QCs: 3.8, 13.1, 24.7	DAY 1			DAY 2						
	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
16W HF F1	7.674	12.7	**19.5**	4.571	18.4	16.8	36.3	**19.5**	76	256
16W HF F2	4.862	24.8	24.1	5.555	19.4	21.6	45.7	22.8	59	387
16W HF F3	3.368	20.8	**14.0**	3.043	19.5	11.9	25.9	**14.0**	65	216
16W HF F4	6.317	22	27.8	9.498	17.2	32.7	60.5	30.2	71	426

16 week low-fluoride males

QCs: 3.4, 14.2, 24.2	DAY 1			DAY 2						
	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
16W LF M1	4.172	10.3	8.6	6.905	16.8	23.2	31.8	15.9	74	215
16W LF M2	7.771	13.7	21.3	6.255	17.7	22.1	43.4	21.7	78	278
16W LF M3	7.198	12.7	**18.3**	3.769	17.6	13.3	31.5	**18.3**	79	231
16W LF M4	5.171	14.0	14.5	7.412	17.7	26.2	40.7	20.4	71	287
16W LF M5	5.619	18.0	20.2	3.851	22.8	17.6	37.8	18.9	81	233
16W LF M6	7.847	18.0	**28.2**	7.760	10.3	16.0	44.2	**28.2**	77	367
16W LF M7	3.792	lost		6.723	16.4	22.1		22.1	77	287
16W LF M8	4.644	15.6	**14.5**	2.531	21.7	11.0	25.5	**14.5**	70	207
16W LF M9	6.516	16.0	20.9	5.823	18.0	21.0	41.8	20.9	67	312

16 week high-fluoride males

QCs: 3.4, 14.2, 24.2	DAY 1			DAY 2						
	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
16W HF M1	5.703	15.0	17.1	7.579	16.5	25.0	42.1	21.1	80	263
16W HF M2	7.946	20.6	**32.7**	5.807	20.8	24.2	56.9	**32.7**	79	414
16W HF M3	6.200	19.1	23.7	5.769	25.0	28.8	52.5	26.3	86	305
16W HF M4	7.228	18.5	26.7	9.288	19.2	35.7	62.4	31.2	71	440
16W HF M5	8.211	20.5	33.7	7.722	22.5	34.7	68.4	34.2	77	444
16W HF M6	7.489	20.0	**30.0**	6.763	15.5	21.0	50.9	**30.0**	71	423
16W HF M7	5.807	21.0	**24.4**	3.912	23.7	18.5	42.9	**24.4**	65	375
16W HF M8	5.619	25.1	**28.2**	4.697	22.6	21.2	49.4	**28.2**	70	403

Table C.32 Laboratory results of urinary levels of aMT6s in female gerbils aged 28 weeks over 48-hours in 2 x 24-hour intervals. Shaded cells indicate where the day 1 levels of aMT6s were used in statistical analyses.

28 week high-fluoride females										
	DAY 1			**DAY 2**						
QCs: 3.6, 13.1, 21.3	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 1**	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 2**	**aMT6s ng/48-h**	**mean aMT6s ng/24-h**	**body wt g**	**aMT6s pg/g BW/24-h**
28W HF F1	6.275	13.5	16.9	6.023	16.0	19.3	36.2	18.1	73	247
28W HF F2	6.275	19.0	23.8	6.344	21.7	27.5	51.4	25.7	64	401
28W HF F3	4.656	17.5	16.3	8.976	9.9	17.8	34.1	17.0	78	219
28W HF F4	6.033	14.4	17.4	3.736	21.6	16.1	33.5	16.8	78	215
28W HF F5	5.292	20.9	22.1	6.849	14.6	20.0	42.1	21.1	74	286
28W HF F6	7.123	14.9	21.2	6.33	20.5	26.0	47.2	23.6	68	345
28W HF F7	4.389	15.2	13.3	3.794	23.0	17.5	30.8	15.4	72	213
28W HF F8	4.551	14.3	13.0	7.831	15.4	24.1	37.1	18.6	81	228
28W HF F9	5.489	16.6	18.2	5.607	17.1	19.2	37.4	18.7	76	248
28W HF F10	11.235	14.0	**31.5**	8.543	15.0	25.6	57.1	**31.5**	78	405
28W HF F11	10.751	15.6	33.5	8.567	18.9	32.4	65.9	33.0	73	450
28W HF F12	4.902	15.5	15.2	4.328	22.7	19.6	34.8	17.4	78	223
28W HF F13	9.275	13.5	**25.0**	6.782	14.0	19.0	44.0	**25.0**	89	280
28 week low-fluoride females										
	DAY 1			**DAY 2**						
QCs: 3.6, 13.1, 21.3	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 1**	**aMT6s ng/ml**	**volume ml**	**aMT6s ng/day 2**	**aMT6s ng/48-h**	**mean aMT6s ng/24-h**	**body wt g**	**aMT6s pg/g BW/24-h**
28W LF F1	6.036	15.5	18.7	7.479	19.5	29.2	47.9	23.9	68	353
28W LF F2	3.83	23.2	17.8	4.511	20.8	18.8	36.5	18.3	67	272
28W LF F3	2.93	25.4	**14.9**	2.890	21.5	12.4	27.3	**14.9**	62	239
28W LF F4	3.978	16.2	12.9	3.970	21.2	16.8	29.7	14.9	64	231
28W LF F5	3.398	20.7	**14.1**	2.382	17.8	8.5	22.5	**14.1**	73	194
28W LF F6	6.099	19.0	**23.2**	4.707	21.0	19.8	42.9	**23.2**	73	319
28W LF F7	4.87	15.2	14.8	4.206	18.3	15.4	30.2	15.1	71	214
28W LF F8	4.934	14.5	14.3	7.360	14.3	21.0	35.4	17.7	72	244
28W LF F9	2.217	16.5	7.3	3.325	16.6	11.0	18.4	9.2	68	135
28W LF F10	2.762	19.0	10.5	3.663	18.2	13.3	23.8	11.9	70	171
28W LF F11	4.306	18.4	15.8	4.591	20.5	18.8	34.7	17.3	69	253
28W LF F12	4.613	21.0	19.4	6.845	14.4	19.7	39.1	19.5	72	272
28W LF F13	7.083	15.5	**22.0**	3.274	19.0	12.4	34.4	**22.0**	79	277
28W LF F14	4.445	21.4	19.0	5.379	23.4	25.2	44.2	22.1	75	294

Table C.33 Laboratory results of urinary levels of aMT6s in male gerbils aged 28 weeks over 48-hours in 2 x 24-hour urine collections. Shaded cells indicate where the day 1 levels of aMT6s were used in statistical analyses.

28 week high-fluoride male gerbils										
	DAY 1			DAY 2						
QCs: 4.1, 14.6, 21.7	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
28W HF M1	10.150	19.5	**39.6**	11.764	13.9	32.7	72.3	**39.6**	93	427
28W HF M2	9.903	12.3	24.4	11.981	12.6	30.2	54.6	27.3	95	286
28W HF M3	8.472	15.0	25.4	12.128	15.2	36.9	62.3	31.1	87	358
28W HF M4	8.274	17.0	28.1	8.834	15.0	26.5	54.6	27.3	83	329
28W HF M5	3.936	15.7	12.4	4.174	18.5	15.4	27.8	13.9	86	162
28W HF M6	5.974	17.3	20.7	9.172	14.0	25.7	46.4	23.2	97	240
28W HF M7	9.314	15.3	28.5	5.220	24.6	25.7	54.2	27.1	103	263
28W HF M8	6.570	20.6	27.1	8.927	21.4	38.2	65.3	32.6	85	384
28 week low-fluoride male gerbils										
	DAY 1			DAY 2						
QCs: 4.1, 14.6, 21.7	aMT6s ng/ml	volume ml	aMT6s ng/day 1	aMT6s ng/ml	volume ml	aMT6s ng/day 2	aMT6s ng/48-h	mean aMT6s ng/24-h	body wt g	aMT6s pg/g BW/24-h
28W LF M1	6.606	17.2	**22.7**	5.775	15.5	17.9	40.6	**22.7**	113	202
28W LF M2	4.689	20.0	18.8	4.646	19.7	18.3	37.1	18.5	108	172
28W LF M3	4.880	17.4	17.0	7.897	16.6	26.2	43.2	21.6	106	204
28W LF M4	4.526	15.0	13.6	5.403	22.5	24.3	37.9	18.9	114	166
28W LF M5	6.173	20.8	25.7	6.099	23.6	28.8	54.5	27.2	73	373
28W LF M6	7.396	20.0	29.6	9.373	18.2	34.1	63.7	31.9	84	379
28W LF M7	7.760	17.4	**27.0**	7.422	13.4	19.9	46.9	**27.0**	77	350
28W LF M8	7.739	13.4	20.7	8.449	14.0	23.7	44.4	22.2	109	203
28W LF M9	7.218	21.5	31.0	6.248	22.8	28.5	59.5	29.8	72	413

Table C.34 Circadian excretion of urinary aMT6s by low-fluoride female gerbils aged 11½ weeks; corrected for intervals when the gerbils did not void; expressed as ng/3-hours.

TIME	11½W LOW-F F1	11½W LOW-F F2	11½W LOW-F F3	11½W LOW-F F4	11½W LOW-F F5	11½W LOW-F F6	11½W LOW-F F7	11½W LOW-F F8	11½W LOW-F F9	11½W LOW-F F10	11½W LOW-F F11	11½W LOW-F F12	MEAN	STDEV	SEM
1300 - 1600	3.6	1.4	1.0	2.2	2.6	2.7	0.9	0.5	0.7	1.7	0.9	0.7	1.6	1.0	0.3
1600 - 1900	2.1	1.3	3.1	3.1	1.4	0.4	0.5	0.5	1.7	1.6	1.0	2.3	1.6	0.9	0.3
1900 - 2200	2.4	1.3	2.1	2.3	1.0	0.4	1.4	1.4	2.0	2.6	1.8	1.1	1.7	0.7	0.2
2200 - 0100	1.7	1.3	1.3	5.8	2.8	0.6	5.9	3.1	2.6	3.5	3.2	2.7	2.9	1.6	0.5
0100 - 0400	17.3	9.2	1.5	12.7	5.9	4.0	5.8	3.1	7.9	3.5	10.8	1.6	6.9	4.8	1.4
0400 - 0700	3.8	1.5	9.9	11.5	9.0	11.7	8.8	12.1	8.2	3.6	3.6	2.6	7.2	3.9	1.1
0700 - 1000	3.7	6.5	0.9	2.9	6.1	3.7	0.6	2.8	1.5	6.4	3.6	3.0	3.5	2.0	0.6
1000 - 1300	2.8	2.6	0.8	1.9	1.5	0.8	0.6	1.4	1.4	2.2	3.5	1.0	1.7	0.9	0.3
1300 - 1600	2.7	2.6	1.6	2.2	1.2	0.5	0.7	1.4	2.5	2.2	3.7	2.2	2.0	0.9	0.3
1600 - 1900	4.1	2.0	2.1	4.6	5.8	1.2	1.0	0.6	1.8	2.8	1.5	1.1	2.4	1.6	0.5
1900 - 2200	3.5	2.1	1.8	1.6	2.5	3.6	1.9	0.6	1.7	3.1	1.8	1.8	2.2	0.9	0.3
2200 - 0100	3.5	4.8	2.5	7.2	1.4	5.0	2.6	0.7	2.4	2.3	1.9	2.0	3.0	1.8	0.5
0100 - 0400	11.2	3.6	3.9	10.6	3.9	10.4	9.0	10.5	2.5	2.3	5.3	1.8	6.3	3.8	1.1
0400 - 0700	6.4	3.6	3.1	7.6	13.0	4.6	9.6	7.3	7.1	10.6	5.4	3.9	6.9	3.0	0.9
0700 - 1000	3.8	5.8	1.6	2.5	3.7	4.4	1.0	1.5	6.1	4.2	8.7	2.7	3.8	2.2	0.6
1000 - 1300	3.2	2.8	3.2	4.6	1.6	1.0	2.2	2.1	2.1	2.0	2.3	1.0	2.3	1.0	0.3
total aMT6s ng/48-h	75.7	52.4	40.4	83.3	63.4	55.0	52.5	49.6	52.1	54.6	59.0	31.5	55.8	13.9	4.0
total aMT6s ng/day 1	37.3	25.1	20.6	42.4	30.3	24.3	24.5	24.9	26.0	25.1	28.4	15.0	27.0	7.2	2.1
total aMT6s ng/day 2	38.4	27.3	19.8	40.9	33.1	30.7	28.0	24.7	26.2	29.5	30.6	16.5	28.8	6.9	2.0
mean aMT6s ng/24-h	37.9	26.2	20.2	41.7	31.7	27.5	26.2	24.8	26.1	27.3	29.5	15.8	27.9	6.9	2.0
body weight g	64	53	58	65	71	64	56	63	53	54	54	56	59	5.9	1.7
mean aMT6s pg/g BW/24-h	591	494	348	641	446	430	469	394	492	506	546	281	470	100	29

Table C.35 Circadian excretion of urinary aMT6s by high-fluoride female gerbils at 11½ weeks; corrected for intervals when gerbils did not void; expressed as ng/3-h.

TIME	11½W HIGH-F F1	11½W HIGH-F F2	11½W HIGH-F F3	11½W HIGH-F F4	11½W HIGH-F F5	11½W HIGH-F F6	11½W HIGH-F F7	11½W HIGH-F F8	11½W HIGH-F F9	11½W HIGH-F F10	11½W HIGH-F F11	11½W HIGH-F F12	MEAN	STDEV	SEM
1300 - 1600	0.8	0.9	0.6	1.1	0.7	0.9	1.7	1.4	1.1	2.1	1.5	1.1	1.2	0.5	0.1
1600 - 1900	3.5	0.8	0.7	2.3	1.8	1.6	1.1	1.5	2.0	1.9	2.3	2.0	1.9	0.7	0.2
1900 - 2200	1.9	1.1	1.4	2.0	0.8	1.9	3.1	1.6	1.9	1.7	1.3	1.1	1.7	0.6	0.2
2200 - 0100	2.2	0.8	5.7	2.2	3.3	2.8	4.9	9.1	1.8	10.7	3.6	1.1	4.3	3.1	0.9
0100 - 0400	7.1	0.9	12.2	10.1	2.6	4.0	4.9	14.9	1.4	13.2	6.9	1.5	7.2	4.8	1.5
0400 - 0700	7.0	1.0	7.0	7.2	1.7	2.8	4.5	5.9	1.8	4.4	7.0	6.0	5.0	2.1	0.6
0700 - 1000	3.5	1.3	4.1	2.6	1.2	1.8	1.4	2.2	0.8	3.6	1.5	3.4	2.4	1.1	0.3
1000 - 1300	3.7	0.8	1.5	2.1	1.7	1.5	2.9	2.3	0.9	1.6	1.4	1.1	1.9	0.8	0.2
1300 - 1600	3.6	0.6	1.5	1.6	1.2	1.0	2.4	0.7	1.5	2.1	3.6	1.1	1.8	1.0	0.3
1600 - 1900	3.4	0.7	1.1	3.0	1.1	1.7	3.5	5.2	2.6	1.9	2.9	4.0	2.8	1.3	0.4
1900 - 2200	2.9	0.9	0.9	2.2	1.0	1.7	1.7	3.3	1.4	1.7	2.7	0.4	1.8	0.9	0.3
2200 - 0100	3.1	0.7	1.2	3.0	3.0	1.1	3.2	2.9	2.3	10.7	4.8	0.4	3.2	2.8	0.8
0100 - 0400	6.7	1.2	7.9	4.4	2.9	3.8	6.4	16.2	1.8	13.2	9.5	0.5	6.7	4.8	1.5
0400 - 0700	8.8	1.5	15.6	4.9	4.3	4.1	2.6	8.4	1.9	4.4	6.3	2.8	5.8	3.9	1.2
0700 - 1000	3.3	1.8	2.0	4.7	1.5	2.6	3.8	3.0	1.4	3.6	2.4	1.5	2.7	1.1	0.3
1000 - 1300	5.1	1.1	1.5	2.3	1.0	1.5	2.6	2.0	1.7	1.6	4.2	3.1	2.4	1.3	0.4
total aMT6s ng/48-h	66.6	16.1	64.9	55.7	29.8	34.8	50.7	80.6	26.3	78.4	62.1	31.1	52.8	19.7	5.9
total aMT6s ng/day 1	29.7	7.6	33.2	29.6	13.8	17.3	24.5	38.9	11.7	39.2	25.6	17.3	25.5	9.6	2.9
total aMT6s ng/day 2	36.9	8.5	31.7	26.1	16.0	17.5	26.2	41.7	14.6	39.2	36.5	13.8	27.3	10.6	3.2
mean aMT6s ng/24-h	33.3	8.1	32.5	27.9	14.9	17.4	25.4	40.3	13.2	39.2	31.0	15.6	26.4	9.9	3.0
body weight g	66	52	67	67	53	60	75	65	60	63	66	54	63	6.3	1.9
mean aMT6s pg/g BW/24-h	505	155	484	416	281	290	338	620	219	622	470	288	412	140	42

N.B. The circadian data for 11½ W HF F10 during day 1 was repeated for day 2 because aMT6s levels in day 1 were substantially higher than those excreted in day 2. No 2 died at 14 weeks and its data at 11½ weeks was not included in statistical analyses.

Table C.36 Circadian excretion of urinary aMT6s by low-fluoride male gerbils aged 11½ weeks; corrected for intervals when gerbils did not void; expressed as ng/3-h.

TIME	11½W LOW-F M1	11½W LOW-F M2	11½W LOW-F M3	11½W LOW-F M4	11½W LOW-F M5	11½W LOW-F M6	11½W LOW-F M7	11½W LOW-F M8	11½W LOW-F M9	11½W LOW-F M10	11½W LOW-F M11	11½W LOW-F M12	MEAN	STDEV	SEM
1300 - 1600	1.6	1.3	1.4	2.3	1.5	0.7	4.6	2.4	1.6	0.7	2.0	1.7	1.8	1.0	0.3
1600 - 1900	1.7	4.9	1.4	0.9	1.5	0.7	1.3	0.6	1.6	0.6	2.6	1.6	1.6	1.2	0.3
1900 - 2200	2.8	3.6	1.9	1.0	2.5	0.7	1.8	1.6	2.3	3.3	2.9	1.5	2.2	0.9	0.3
2200 - 0100	3.9	3.6	2.0	3.9	3.7	0.9	10.1	2.5	3.9	3.8	2.0	3.1	3.6	2.3	0.7
0100 - 0400	7.1	5.8	8.3	14.0	10.6	5.9	16.8	13.5	1.8	6.2	1.1	2.2	7.8	5.1	1.5
0400 - 0700	7.1	6.4	8.3	14.0	11.2	6.1	7.3	10.5	11.4	5.1	5.1	11.1	8.6	2.9	0.8
0700 - 1000	8.8	1.8	2.3	1.9	1.9	2.5	7.2	11.0	4.7	2.5	4.5	5.4	4.5	3.1	0.9
1000 - 1300	3.2	1.7	2.3	1.9	1.3	1.4	5.1	2.6	1.5	2.4	3.2	1.4	2.3	1.1	0.3
1300 - 1600	3.1	1.3	3.5	1.9	2.5	1.3	4.0	2.1	2.6	3.0	2.8	3.6	2.6	0.9	0.3
1600 - 1900	2.5	4.9	3.6	3.4	1.7	0.9	4.0	2.8	3.1	1.6	1.6	3.1	2.8	1.2	0.3
1900 - 2200	2.9	3.6	2.1	1.8	1.8	0.5	2.9	3.3	3.0	3.3	3.4	1.4	2.5	1.0	0.3
2200 - 0100	1.5	3.6	3.8	1.7	6.7	1.4	2.3	2.9	2.5	2.4	2.9	1.4	2.8	1.5	0.4
0100 - 0400	3.8	5.8	8.2	13.9	9.3	3.9	12.6	16.9	5.5	2.3	3.3	7.5	7.8	4.6	1.3
0400 - 0700	16.0	6.4	8.2	10.3	8.3	10.8	19.6	8.8	5.4	3.7	3.2	7.3	9.0	4.8	1.4
0700 - 1000	1.7	1.8	4.1	4.9	5.1	1.8	7.7	2.9	2.9	3.8	6.0	3.3	3.8	1.8	0.5
1000 - 1300	3.1	1.7	1.5	2.7	1.6	1.0	3.4	2.2	2.7	3.5	1.9	1.1	2.2	0.9	0.3
total aMT6s ng/48-h	70.8	58.2	62.9	80.5	71.2	40.5	110.7	86.6	56.5	48.2	48.5	56.7	65.9	19.5	5.6
total aMT6s ng/day 1	36.2	29.1	27.9	39.9	34.2	18.9	54.2	44.7	28.8	24.6	23.4	28.0	32.5	9.9	2.9
total aMT6s ng/day 2	34.6	29.1	35.0	40.6	37.0	21.6	56.5	41.9	27.7	23.6	25.1	28.7	33.5	9.8	2.8
mean aMT6s ng/24-h	35.4	29.1	31.5	40.3	35.6	20.3	55.4	43.3	28.3	24.1	24.3	28.4	33.0	9.8	2.8
body weight g	78	71	74	70	69	68	86	76	69	84	71	62	73	6.9	2.0
mean aMT6s pg/g BW/24-h	454	410	425	575	516	298	644	570	409	287	342	457	449	112	32

N.B. The circadian data obtained during day 1 was repeated for day 2 for 11½ WLF M2 because the levels of urinary aMT6s were substantially higher (CV% > 10%) than those excreted in day 2 (shaded cells).

Table C.37 Circadian excretion of urinary aMT6s by high-fluoride male gerbils aged 11½ weeks; corrected for intervals when gerbils did not void; expressed as ng/3-h.

TIME	11½W HIGH-F M1	11½W HIGH-F M2	11½W HIGH-F M3	11½W HIGH-F M4	11½W HIGH-F M5	11½W HIGH-F M6	11½W HIGH-F M7	11½W HIGH-F M8	11½W HIGH-F M9	11½W HIGH-F M10	11½W HIGH-F M11	11½W HIGH-F M12	MEAN	STDEV	SEM
1300 - 1600	1.7	1.1	0.7	0.5	1.9	2.0	0.8	0.9	0.5	1.2	0.5	1.0	1.1	0.5	0.2
1600 - 1900	1.6	0.7	2.0	0.5	1.7	2.1	0.9	1.0	1.8	1.3	0.5	1.0	1.3	0.6	0.2
1900 - 2200	2.0	4.7	1.1	1.4	3.1	0.9	1.5	2.1	1.9	2.0	3.2	2.8	2.2	1.1	0.3
2200 - 0100	3.0	4.7	0.6	1.5	3.0	0.9	3.1	4.0	4.3	6.3	2.3	4.6	3.2	1.7	0.5
0100 - 0400	2.6	4.7	3.3	0.8	4.9	6.1	6.6	8.9	4.4	6.4	3.5	3.4	4.6	2.2	0.6
0400 - 0700	3.3	1.3	1.0	1.8	3.5	5.7	3.5	5.5	4.4	3.4	5.1	3.5	3.5	1.5	0.4
0700 - 1000	3.3	2.3	3.0	2.1	3.5	5.6	1.8	1.8	0.8	3.3	1.4	3.5	2.7	1.3	0.4
1000 - 1300	3.5	1.9	1.9	1.2	4.0	1.2	1.7	1.8	0.7	4.2	1.2	1.4	2.1	1.2	0.3
1300 - 1600	1.2	1.9	0.6	1.1	1.3	1.1	**0.8**	3.1	**0.5**	**1.2**	0.7	1.4	1.2	0.7	0.2
1600 - 1900	1.1	1.9	0.7	1.2	1.3	1.8	**0.9**	4.4	**1.8**	**1.3**	0.6	1.4	1.5	1.0	0.3
1900 - 2200	2.5	2.2	1.7	1.1	2.4	1.7	**1.5**	2.6	**1.9**	**2.0**	3.3	1.4	2.0	0.6	0.2
2200 - 0100	5.9	6.5	0.8	1.0	4.6	1.6	**3.1**	2.4	**4.3**	**6.3**	1.5	5.9	3.7	2.2	0.6
0100 - 0400	6.0	6.6	3.8	3.1	4.6	1.7	**6.6**	2.4	**4.4**	**6.4**	5.9	5.1	4.7	1.7	0.5
0400 - 0700	3.4	6.7	3.7	2.7	8.1	9.5	**3.5**	9.8	**4.4**	**3.4**	4.1	6.2	5.5	2.5	0.7
0700 - 1000	3.4	2.3	1.0	0.8	2.7	4.3	**1.8**	6.2	**0.8**	**3.3**	2.2	1.9	2.6	1.6	0.5
1000 - 1300	1.4	2.2	1.3	1.8	4.5	1.2	**1.7**	2.2	**0.7**	**4.2**	2.7	1.9	2.2	1.2	0.3
total aMT6s ng/48-h	45.9	51.7	27.2	22.6	55.1	47.4	39.8	59.1	37.6	56.2	38.7	46.4	44.0	11.3	3.3
total aMT6s ng/day 1	21.0	21.4	13.6	9.8	25.6	24.5	19.9	26.0	18.8	28.1	17.7	21.2	20.6	5.3	1.5
total aMT6s ng/day 2	24.9	30.3	13.6	12.8	29.5	22.9	19.9	33.1	18.8	28.1	21.0	25.2	23.3	6.4	1.9
mean aMT6s ng/24-h	23.0	25.9	13.6	11.3	27.6	23.7	19.9	29.6	18.8	28.1	19.4	23.2	22.0	5.7	1.6
body weight g	77	75	72	66	71	70	74	80	71	71	77	72	73	3.8	1.1
mean aMT6s pg/g BW/24-h	298	345	189	171	388	339	269	369	265	396	251	322	300	73	21

N.B. The circadian data obtained during day 1 were repeated for day 2 for 11½ W HF M7, M9 and M10 because the levels of urinary aMT6s in day 1 were substantially higher (CV% > 10%) than those excreted in day 2 (shaded cells).

Table C.38 Circadian excretion of aMT6s from low-F female gerbils aged 11½ weeks; corrected for non-urinations, expressed as pg/g BW/3-hours

TIME	11½W LOW-F F1	11½W LOW-F F2	11½W LOW-F F3	11½W LOW-F F4	11½W LOW-F F5	11½W LOW-F F6	11½W LOW-F F7	11½W LOW-F F8	11½W LOW-F F9	11½W LOW-F F10	11½W LOW-F F11	11½W LOW-F F12	MEAN	STDEV	SEM
1300 - 1600	56	25	17	34	37	42	16	8	13	31	17	13	26	15	4
1600 - 1900	33	25	53	48	20	6	9	8	32	30	19	41	27	16	4
1900 - 2200	38	25	36	35	14	6	25	22	38	48	33	20	28	12	3
2200 - 0100	27	25	22	89	39	9	104	49	49	65	59	48	49	28	8
0100 - 0400	270	174	26	195	83	63	104	49	149	65	200	29	117	79	23
0400 - 0700	59	28	171	177	127	183	157	192	155	67	66	46	119	61	18
0700 - 1000	58	123	16	45	86	58	11	44	27	119	67	54	59	36	10
1000 - 1300	43	49	14	29	21	13	11	22	26	41	65	18	29	17	5
1300 - 1600	42	49	28	34	17	8	13	22	47	41	69	39	34	17	5
1600 - 1900	64	38	36	71	82	19	18	10	33	52	28	20	39	23	7
1900 - 2200	55	40	31	25	35	56	34	10	32	57	33	32	37	14	4
2200 - 0100	55	91	43	111	20	78	46	11	45	43	35	36	51	29	8
0100 - 0400	175	68	67	163	55	163	161	167	47	43	98	32	103	57	17
0400 - 0700	100	68	53	117	183	72	171	116	134	196	100	70	115	48	14
0700 - 1000	59	109	28	38	52	69	18	24	115	78	161	48	67	43	12
1000 - 1300	50	53	55	71	23	16	39	33	40	37	43	18	40	16	5
total aMT6s pg/g BW/48-h	1183	988	697	1282	893	859	937	788	983	1012	1092	563	940	200	58
total aMT6s pg/g BW/day 1	583	473	355	652	427	380	437	395	490	465	525	268	454	103	30
total aMT6s pg/g BW/day 2	600	515	341	629	466	480	500	393	493	546	567	295	485	101	29
mean aMT6s pg/g BW/24-h	591	494	348	641	446	430	469	394	492	506	546	281	470	100	29

Table C.39 Circadian excretion of aMT6s by high-F female gerbils aged 11½ weeks; corrected for non-urinations, expressed as pg/g BW/3-h. No 2 died at 14 weeks and its data at 11½ weeks were not included in statistical analyses.

TIME	11½W HIGH-F F1	11½W HIGH-F F2	11½W HIGH-F F3	11½W HIGH-F F4	11½W HIGH-F F5	11½W HIGH-F F6	11½W HIGH-F F7	11½W HIGH-F F8	11½W HIGH-F F9	11½W HIGH-F F10	11½W HIGH-F F11	11½W HIGH-F F12	MEAN	STDEV	SEM
1300 - 1600	12	17	9	16	13	15	23	22	18	33	23	20	19	7	2
1600 - 1900	53	15	10	34	34	27	15	23	33	30	35	37	30	12	3
1900 - 2200	29	21	21	30	15	32	41	25	32	27	20	20	27	7	2
2200 - 0100	33	15	85	33	62	47	65	140	30	170	55	20	67	48	14
0100 - 0400	108	17	182	151	49	67	65	229	23	210	105	28	111	73	21
0400 - 0700	106	19	104	107	32	47	60	91	30	70	106	111	79	32	9
0700 - 1000	53	25	61	39	23	30	19	34	13	57	23	63	38	18	5
1000 - 1300	56	15	22	31	32	25	39	35	15	25	21	20	29	11	3
1300 - 1600	55	12	22	24	23	17	32	11	25	33	55	20	29	14	4
1600 - 1900	52	13	16	45	21	28	47	80	43	30	44	74	44	20	6
1900 - 2200	44	17	13	33	19	28	23	51	23	27	41	7	28	13	4
2200 - 0100	47	13	18	45	57	18	43	45	38	170	73	7	51	44	13
0100 - 0400	102	23	118	66	55	63	85	249	30	210	144	9	103	74	21
0400 - 0700	133	29	233	73	81	68	35	129	32	70	95	52	91	57	17
0700 - 1000	50	35	30	70	28	43	51	46	23	57	37	28	42	14	4
1000 - 1300	77	21	22	34	19	25	35	31	28	25	64	57	38	19	6
total aMT6s pg/g BW/48-h	1009	310	969	831	562	580	676	1240	438	1244	940	576	824	279	81
total aMT6s pg/g BW/day 1	450	146	496	442	260	288	327	598	195	622	388	320	399	137	40
total aMT6s pg/g BW/day 2	559	163	473	390	302	292	349	642	243	622	553	256	425	150	43
mean aMT6s pg/g BW/24-h	505	155	484	416	281	290	338	620	219	622	470	288	412	140	40

N.B. The circadian data obtained during day 1 were repeated for day 2 for 11½ week HF F10 because the levels of urinary aMT6s were substantially higher (CV% > 10%) than those excreted in day 2 (shaded cells).

Table C.40 Circadian excretion of aMT6s from low-F male gerbils at 11½ weeks; corrected for non-urinations; expressed as pg/g BW/3-h.

TIME	11½W LOW-F M1	11½W LOW-F M2	11½W LOW-F M3	11½W LOW-F M4	11½W LOW-F M5	11½W LOW-F M6	11½W LOW-F M7	11½W LOW-F M8	11½W LOW-F M9	11½W LOW-F M10	11½W LOW-F M11	11½W LOW-F M12	MEAN	STDEV	SEM
1300 - 1600	21	18	19	33	22	10	53	32	23	8	28	27	25	12	3
1600 - 1900	22	69	19	13	22	10	15	8	23	7	37	26	23	17	5
1900 - 2200	36	51	26	14	36	10	21	21	33	39	41	24	29	12	3
2200 - 0100	50	51	27	56	54	13	117	33	57	45	28	50	48	26	7
0100 - 0400	91	82	112	200	154	87	195	178	26	74	15	35	104	65	19
0400 - 0700	91	90	112	200	162	90	85	138	165	61	72	179	120	47	13
0700 - 1000	113	25	31	27	28	37	84	145	68	30	63	87	61	39	11
1000 - 1300	41	24	31	27	19	21	59	34	22	29	45	23	31	12	3
1300 - 1600	40	18	47	27	36	19	47	28	38	36	39	58	36	12	3
1600 - 1900	32	69	49	49	25	13	47	37	45	19	23	50	38	16	5
1900 - 2200	37	51	28	26	26	7	34	43	43	39	48	23	34	12	4
2200 - 0100	19	51	51	24	97	21	27	38	36	29	41	23	38	22	6
0100 - 0400	49	82	111	199	135	57	147	222	80	27	46	121	106	61	18
0400 - 0700	205	90	111	147	120	159	228	116	78	44	45	118	122	57	16
0700 - 1000	22	25	55	70	74	26	90	38	42	45	85	53	52	23	7
1000 - 1300	40	24	20	39	23	15	40	29	39	42	27	18	30	10	3
total aMT6s pg/g BW/48-h	908	820	850	1150	1032	596	1287	1139	819	574	683	915	898	223	64
total aMT6s pg/g BW/day 1	464	410	377	570	496	278	630	588	417	293	330	452	442	115	33
total aMT6s pg/g BW/day 2	444	410	473	580	536	318	657	551	401	281	354	463	456	112	32
mean aMT6s pg/g BW/24-h	454	410	425	575	516	298	644	570	409	287	342	457	449	112	32

N.B. The circadian data obtained during day 1 were repeated for day 2 for 11½ week LF M2 because the levels of urinary aMT6s were substantially higher (CV% > 10%) in day 1 than those excreted in day 2 (shaded cells).

Table C.41 Circadian excretion of aMT6s by high-F male gerbils at 11½ weeks; corrected for non-urinations; expressed as pg/g BW/3-h.

TIME	11½W HF M1	11½W HF M2	11½W HF M3	11½W HF M4	11½W HF M5	11½W HF M6	11½W HF M7	11½W HF M8	11½W HF M9	11½W HF M10	11½W HF M11	11½W HF M12	MEAN	STDEV	SEM
1300 - 1600	22	15	10	8	27	29	11	11	7	17	6	14	15	8	2
1600 - 1900	21	9	28	8	24	30	12	13	25	18	6	14	17	8	2
1900 - 2200	26	63	15	21	44	13	20	26	27	28	42	39	30	14	4
2200 - 0100	39	63	8	23	42	13	42	50	61	89	30	64	44	23	7
0100 - 0400	34	63	46	12	69	87	89	111	62	90	45	47	63	28	8
0400 - 0700	43	17	14	27	49	81	47	69	62	48	66	49	48	21	6
0700 - 1000	43	31	42	32	49	80	24	23	11	46	18	49	37	18	5
1000 - 1300	45	25	26	18	56	17	23	23	10	59	16	19	28	16	5
1300 - 1600	16	25	8	17	18	16	**11**	39	**7**	**17**	9	19	17	9	3
1600 - 1900	14	25	10	18	18	26	**12**	55	**25**	**18**	8	19	21	12	4
1900 - 2200	32	29	24	17	34	24	**20**	33	**27**	**28**	43	19	28	7	2
2200 - 0100	77	87	11	15	65	23	**42**	30	**61**	**89**	19	82	50	30	9
0100 - 0400	78	88	53	47	65	24	**89**	30	**62**	**90**	77	71	64	22	6
0400 - 0700	44	89	51	41	114	136	**47**	123	**62**	**48**	53	86	75	34	10
0700 - 1000	44	31	14	12	38	61	**24**	78	**11**	**46**	29	26	35	20	6
1000 - 1300	18	29	18	27	63	17	**23**	28	**10**	**59**	35	26	30	16	5
total aMT6s pg/g BW/48-h	596	689	378	342	776	677	538	739	530	792	503	644	600	147	42
total aMT6s pg/g BW/day 1	273	285	189	148	361	350	269	325	265	396	230	294	282	71	21
total aMT6s pg/g BW/day 2	323	404	189	194	415	327	269	414	265	396	273	350	318	81	23
mean aMT6s pg/g BW/24-h	298	345	189	171	388	339	269	369	265	396	251	322	300	73	21

N.B. The circadian data obtained during day 1 were repeated for day 2 for 11½ week HF M7, M9 and M10 because the levels of urinary aMT6s in day 1 were substantially higher (CV% > 10%) than those excreted in day 2 (shaded cells).

Table C.42 Circadian excretion of urinary aMT6s by low-F female gerbils aged 16 weeks; corrected for non-urinations; data expressed as ng/3-h.

WINTER TIME	16W LF F1	16W LF F2	16W LF F3	16W LF F4	16W LF F5	16W LF F6	16W LF F7	16W LF F8	16W LF F9	16W LF F10	16W LF F11	16W LF F12	MEAN	STDEV	SEM
1800 - 2100	1.9	5.6	0.8	1.6	1.4	3.4	2.0	1.2	1.2	3.4	2.4	2.2	2.3	1.3	0.4
2100 - 2400	4.9	5.8	1.9	3.3	1.5	2.1	2.6	1.3	1.2	1.5	2.5	0.9	2.5	1.5	0.4
2400 - 0300	9.6	6.2	2.5	7.2	3.9	5.4	8.1	9.5	7.8	3.9	2.4	1.4	5.7	2.8	0.8
0300 - 0600	9.7	6.2	3.9	7.2	14.2	7.1	15.3	5.3	8.6	6.2	15.9	3.0	8.6	4.4	1.3
0600 - 0900	2.6	8.9	2.0	7.3	3.2	2.5	3.3	1.2	2.5	6.1	2.7	1.8	3.7	2.4	0.7
0900 - 1200	2.7	3.7	1.8	2.2	4.0	3.2	2.2	1.1	2.4	5.6	2.7	1.1	2.7	1.3	0.4
1200 - 1500	2.4	1.9	1.9	2.2	1.9	1.0	1.6	3.9	2.7	1.9	1.6	2.4	2.1	0.7	0.2
1500 - 1800	2.4	2.0	1.3	3.4	4.9	3.9	2.6	1.2	2.5	2.0	1.6	1.4	2.4	1.1	0.3
total aMT6s ng/24-h	36.2	40.3	16.1	34.4	35.0	28.6	37.7	24.7	28.9	30.6	31.8	14.2	29.9	8.1	2.3
body weight g	68	61	63	77	75	69	68	75	63	64	62	66	68	5.5	1.6
aMT6s pg/g BW/24-h	532	661	256	447	467	414	554	329	459	478	513	215	444	126	36

Table C.43 Circadian excretion of urinary aMT6s by low-F female gerbils aged 16 weeks; corrected for non-urinations; data expressed as pg/g BW/3-h.

WINTER TIME	16W LF F1	16W LF F2	16W LF F3	16W LF F4	16W LF F5	16W LF F6	16W LF F7	16W LF F8	16W LF F9	16W LF F10	16W LF F11	16W LF F12	MEAN	STDEV	SEM
1800 - 2100	28	92	13	21	19	50	29	16	19	53	39	33	34	22	6
2100 - 2400	72	95	30	43	20	30	38	17	18	23	40	13	37	24	7
2400 - 0300	141	102	39	94	52	79	119	126	124	61	39	21	83	40	12
0300 - 0600	143	102	62	94	189	103	225	71	124	97	256	46	126	66	19
0600 - 0900	38	146	32	95	43	36	48	16	40	95	44	27	55	37	11
0900 - 1200	40	61	29	29	54	46	32	15	38	88	44	16	41	20	6
1200 - 1500	35	31	30	29	26	15	24	52	43	30	26	36	31	10	3
1500 - 1800	35	33	21	44	65	57	38	16	40	31	26	21	36	15	4
total aMT6s pg/g BW/24-h	533	661	256	447	467	415	552	329	446	477	513	237	444	123	36

Table C.44 Circadian excretion of urinary aMT6s by high-F female gerbils at 16 weeks; corrected for non-urinations; data expressed as aMT6s ng/3-h.

TIME	16W HF F1	16W HF F2	16W HF F3	16W HF F4	16W HF F5	16W HF F6	16W HF F7	16W HF F8	16W HF F9	16W HF F10	16W HF F11	16W HF F12	MEAN	SD	SEM
1600 - 1900	2.3	2.8	2.8	2.1	1.1	0.3	2.6	1.2	0.4	1.2	0.9	0.4	1.5	1.0	0.3
1900 - 2200	2.4	1.4	2.6	2.1	2.2	0.6	5.0	1.9	0.5	0.9	0.9	0.4	1.7	1.3	0.4
2200 - 0100	5.2	1.4	1.9	3.0	2.2	0.3	5.0	9.0	0.4	5.8	2.4	1.8	3.2	2.6	0.7
0100 - 0400	5.2	5.3	7.3	2.9	3.0	1.2	1.3	8.9	0.5	13.2	6.4	1.7	4.7	3.8	1.1
0400 - 0700	11.2	5.4	13.5	12.2	3.0	1.7	8.0	7.9	1.5	9.1	4.8	3.5	6.8	4.1	1.2
0700 - 1000	2.3	4.0	1.7	2.0	1.3	1.4	8.1	7.9	2.0	2.9	1.2	3.4	3.2	2.4	0.7
1000 - 1300	2.5	2.0	1.8	2.2	1.9	0.7	6.1	1.7	1.0	1.5	2.7	2.2	2.2	1.4	0.4
1300 - 1600	2.0	1.9	1.7	1.4	1.2	1.2	2.5	1.7	1.0	1.5	1.5	0.9	1.5	0.4	0.1
total aMT6s ng/24-h	33.1	24.2	33.3	27.9	15.9	7.4	38.6	40.2	7.1	36.1	20.7	14.3	24.9	11.8	3.4
body weight g	69.4	65.6	69.4	70.4	58.3	48.6	90.8	70.3	62.1	70.7	73.5	65.9	68	9.9	2.9
aMT6s pg/g BW/24-h	477	369	480	396	273	153	425	572	115	510	282	216	356	147	43

Table C.45 Circadian excretion of urinary aMT6s by high-F female gerbils at 16 weeks; corrected for non-urinations; data expressed as pg/g BW/3-h.

TIME	16W HF F1	16W HF F2	16W HF F3	16W HF F4	16W HF F5	16W HF F6	16W HF F7	16W HF F8	16W HF F9	16W HF F10	16W HF F11	16W HF F12	MEAN	SD	SEM
1600 - 1900	33	42	41	30	19	6	29	18	6	17	13	6	22	13	4
1900 - 2200	35	21	38	30	38	13	55	27	8	12	12	6	25	15	4
2200 - 0100	75	21	28	43	38	6	55	129	6	82	32	27	45	35	10
0100 - 0400	75	80	106	41	52	24	14	127	7	186	86	26	69	53	15
0400 - 0700	162	82	196	174	52	34	88	113	24	128	65	53	98	57	16
0700 - 1000	33	61	25	29	22	28	89	113	32	41	16	52	45	29	8
1000 - 1300	36	30	26	31	33	15	67	24	16	21	36	33	31	13	4
1300 - 1600	29	29	24	20	21	25	27	24	16	21	20	14	23	5	1
aMT6s pg/g BW/24-h	480	367	483	399	259	152	424	575	115	508	280	216	355	149	43

Table C.46 Circadian excretion of urinary aMT6s by low-F male gerbils at 16 weeks; corrected for non-urinations; data expressed as aMT6s ng/3-h.

WINTER TIME	16W LF M1	16W LF M2	16W LF M3	16W LF M4	16W LF M5	16W LF M6	16W LF M7	16W LF M8	16W LF M9	16W LF M10	16W LF M11	16W LF M12	MEAN	SD	SEM
1800 - 2100	1.6	2.5	1.4	5.9	2.3	1.3	2.9	2.3	3.4	2.6	3.3	3.7	2.8	1.3	0.4
2100 - 2400	1.6	4.7	4.1	6.4	2.4	1.1	4 8	4.1	0.9	2.4	2.3	4.0	3.2	1.7	0.5
2400 - 0300	8.5	7.0	15.7	8.8	7.5	2.2	10.8	12.9	3.6	4.3	1.5	4.1	7.2	4.4	1.3
0300 - 0600	10.2	7.0	6.3	8.9	13.7	2.7	17.7	23.3	3.5	5.0	4.0	4.2	8.9	6.4	1.8
0600 - 0900	3.4	2.7	3.8	14.1	3.8	1.7	6.5	3.6	3.5	2.6	2.6	4.1	4.4	3.3	0.9
0900 - 1200	2.1	2.7	2.6	1.8	1.6	1.2	2.8	2.7	2.2	1.4	1.8	2.4	2.1	0.5	0.2
1200 - 1500	2.0	2.3	2.6	1.7	2.6	2.0	2.7	2.7	1.7	1.3	3.1	2.4	2.3	0.5	0.2
1500 - 1800	3.7	2.4	2.5	5.4	3.3	1.2	5.9	4.8	2.8	1.8	1.1	2.9	3.2	1.6	0.4
total aMT6s ng/24-h	33.0	31.3	39.1	53.4	37.6	13.4	54.2	56.4	21.6	21.5	19.7	27.9	34.1	14.5	4.2
body weight g	88	82	88	82	81	74	108	91	81	91	76	73	85	9.6	2.8
aMT6s pg/g BW/24-h	375	382	444	651	464	181	502	620	267	236	259	382	397	148	43

Table C.47 Circadian excretion of urinary aMT6s by low-F male gerbils at 16 weeks; corrected for non-urinations; data expressed as pg/g BW/3-h.

WINTER TIME	16W LF M1	16W LF M2	16W LF M3	16W LF M4	16W LF M5	16W LF M6	16W LF M7	16W LF M8	16W LF M9	16W LF M10	16W LF M11	16W LF M12	MEAN	SD	SEM
1800 - 2100	18	30	16	72	28	18	27	25	42	29	43	51	33	16.3	4.7
2100 - 2400	18	57	47	78	30	15	44	45	11	26	30	55	38	19.9	5.7
2400 - 0300	97	85	178	107	93	30	100	142	44	47	20	56	83	46.8	13.5
0300 - 0600	116	85	72	109	169	36	164	256	43	55	53	58	101	66.0	19.0
0600 - 0900	39	33	43	172	47	23	60	40	43	29	34	56	52	39.4	11.4
0900 - 1200	24	33	30	22	20	16	26	30	27	15	24	33	25	5.9	1.7
1200 - 1500	23	28	30	21	32	27	25	30	21	14	41	33	27	6.9	2.0
1500 - 1800	42	29	28	66	41	16	55	53	35	20	14	40	37	16.0	4.6
aMT6s pg/g BW/24-h	375	382	444	651	464	181	502	620	267	236	259	382	397	148	42.8

Table C.48 Circadian excretion of urinary aMT6s by high-F male gerbils at 16 weeks; corrected for non-urinations; data expressed as aMT6s ng/3-h.

TIME	16W HF M1	16W HF M2	16W HF M3	16W HF M4	16W HF M5	16W HF M6	16W HF M7	16W HF M8	16W HF M9	16W HF M10	16W HF M11	16W HF M12	MEAN	SD	SEM
1600 - 1900	2.2	2.4	1.6	1.6	2.8	1.6	2.8	2.6	2.0	2.7	1.6	1.4	2.1	0.5	0.2
1900 - 2200	2.2	4.6	1.3	1.7	4.3	2.3	1.6	4.2	2.1	3.8	1.7	3.2	2.7	1.2	0.3
2200 - 0100	6.5	12.3	2.0	2.0	2.6	2.7	1.6	5.0	2.2	6.8	2.6	3.3	4.1	3.1	0.9
0100 - 0400	6.5	12.3	3.8	3.2	2.6	2.8	11.0	5.1	2.2	6.8	5.9	10.2	6.0	3.5	1.0
0400 - 0700	6.5	9.2	4.8	10.1	13.4	2.7	6.5	15.0	12.6	3.3	5.8	10.2	8.3	4.0	1.2
0700 - 1000	2.3	9.7	2.5	2.1	2.4	2.7	1.5	6.4	1.7	3.3	1.6	9.7	3.8	3.0	0.9
1000 - 1300	2.3	3.6	2.6	1.8	3.7	2.2	1.5	2.5	1.7	2.2	1.9	2.0	2.3	0.7	0.2
1300 - 1600	2.3	3.5	2.1	1.9	2.3	1.5	2.7	2.5	1.5	2.1	1.8	2.0	2.2	0.6	0.2
total aMT6s ng/24-h	30.8	57.6	20.7	24.3	34.1	18.5	29.2	43.3	26.0	31.0	22.9	42.0	31.7	11.2	3.2
body weight g	78	86	75	69	78	68	76	86	80	87	76	80	78	6.1	1.8
aMT6s pg/g BW/24-h	395	670	276	353	437	272	384	503	325	356	301	525	400	118	34

Table C.49 Circadian excretion of urinary aMT6s by high-F male gerbils at 16 weeks; corrected for non-urinations; data expressed as pg/g BW/3-h.

TIME	16W HF M1	16W HF M2	16W HF M3	16W HF M4	16W HF M5	16W HF M6	16W HF M7	16W HF M8	16W HF M9	16W HF M10	16W HF M11	16W HF M12	MEAN	SD	SEM
1600 - 1900	28	28	21	24	36	24	37	30	25	31	21	18	27	6	2
1900 - 2200	28	53	17	25	55	34	21	49	26	44	22	40	35	13	4
2200 - 0100	83	143	27	28	33	40	21	58	28	78	34	41	51	35	10
0100 - 0400	83	143	51	46	33	41	145	59	28	78	78	128	76	42	12
0400 - 0700	83	107	63	146	172	40	86	174	158	38	76	128	106	49	14
0700 - 1000	29	113	33	31	31	40	20	74	21	38	21	121	48	35	10
1000 - 1300	29	42	35	26	47	32	20	29	21	25	25	25	30	8	2
1300 - 1600	29	41	28	27	29	22	36	29	19	24	24	25	28	6	2
aMT6s pg/g BW/24-h	395	670	276	353	437	272	384	503	325	356	301	525	400	118	34

Table D.1 Descriptive statistics for urinary aMT6s levels by gerbils at 7 weeks.

	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
Females	7W LF FEMALES	7W HF FEMALES	7W LF FEMALES	7W HF FEMALES	7W LF FEMALES	7W HF FEMALES
Mean	26.8	18.1	602	360	45	51
Standard Error	2.0	1.6	49	32	0.9	1.6
Median	26.3	15.3	545	292	45	53
Mode	37.0	#N/A	#N/A	#N/A	42	53
Standard Deviation	6.8	5.5	168	109	3.0	5.6
Variance	46.7	30.1	28361	11901	9.2	31
Kurtosis	-1.1	-0.4	-1.1	-1.5	4.6	0.3
Skewness	0.5	1.1	0.6	0.6	1.9	-1.1
Range	19.3	15.4	490	280	11	18
Minimum	17.7	12.2	386	259	42	40
Maximum	37.0	27.6	876	539	53	58
Sum	321	217	7221	4314	538	609
Count	12	12	12	12	12	12
Males	7W LF MALES	7W HF MALES	7W LF MALES	7W HF MALES	7W LF MALES	7W HF MALES
Mean	30.7	16.4	566	308	54	54
Standard Error	2.3	1.2	42	22	0.9	1.7
Median	30.8	15.3	576	306	54	55
Mode	#N/A	#N/A	#N/A	#N/A	50	56.6
Standard Deviation	7.9	4.2	147	76	3.0	5.8
Variance	62	18	21533	5753	9	34
Kurtosis	-1.8	7.0	-1.1	3.2	-1.1	-0.4
Skewness	0.0	2.4	0.3	1.1	0.1	0.1
Range	21.5	16.5	455	311	9	20
Minimum	20.0	12.0	381	185	50	45
Maximum	41.5	28.5	836	496	59	65
Sum	368	197	6792	3699	649	648
Count	12	12	12	12	12	12

Table D.2 Descriptive statistics for urinary aMT6s levels by gerbils at 9 weeks; including 2 gerbils whose day 1 levels of aMT6s were used in analyses.

	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
Females	9W LF FEMALES	9W HF FEMALES	9W LF FEMALES	9W HF FEMALES	9W LF FEMALES	9W HF FEMALES
Mean	25.7	20.2	476	365	54	55
Standard Error	1.8	1.8	31	31	1.0	1.1
Median	25.1	18.5	459	340	53	55
Mode	#N/A	#N/A	#N/A	#N/A	52	54
Standard Deviation	6.4	6.2	109	106	3.4	4.0
Variance	41.0	38.3	11853	11214	11.6	16
Kurtosis	0.3	-1.1	1.2	-1.0	-0.8	-0.3
Skewness	-0.5	0.5	-0.7	0.6	0.6	-0.1
Range	22.2	17.9	411	310	10	13
Minimum	12.3	12.0	233	242	50	48
Maximum	34.4	29.9	644	552	60	61
Sum	309	242	5708	4381	648	662
Count	12	12	12	12	12	12
Males	9W LF MALES	9W HF MALES	9W LF MALES	9W HF MALES	9W LF MALES	9W HF MALES
Mean	27.9	19.6	425	320	66	61
Standard Error	2.2	1.4	32.6	21.5	1.4	1.0
Median	25.8	19.3	410	299	65	61
Mode	#N/A	15.7	#N/A	#N/A	63.8	60
Standard Deviation	7.7	4.7	113	75	4.9	3.5
Variance	58.8	22.4	12785	5568	24.1	12.4
Kurtosis	-0.6	1.4	-0.7	0.3	-0.4	-0.6
Skewness	0.6	1.1	0.4	0.8	0.7	-0.1
Range	24.4	17.3	376	263	15	12
Minimum	18.4	13.3	252	213	60	55
Maximum	42.8	30.6	628	476	75	67
Sum	335	235	5099	3841	790	736
Count	12	12	12	12	12	12

Table D.3 Descriptive statistics for the urinary aMT6s levels excreted in 24-h by gerbils aged 11½ weeks (including 5 gerbils whose day 1 levels of aMT6s were used).

Females	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
	11½ W LF FEMALES	11½ W HF FEMALES	11½ W LF FEMALES	11½ W HF FEMALES	11½ W LF FEMALES	11½ W HF FEMALES
Mean	27.7	26.1	467	407	59	63
Standard Error	2.0	2.9	29	40	1.7	1.9
Median	26.3	27.8	478	413	57	65
Mode	#N/A	#N/A	#N/A	#N/A	64	66
Standard Deviation	7.1	9.5	102	133	5.9	6.3
Variance	49.8	90.2	10340	17612	35.1	40
Kurtosis	0.8	-1.6	0.2	-1.3	-0.7	0.3
Skewness	0.4	-0.1	-0.2	0.2	0.6	-0.1
Range	26.5	26.3	369	387	18	22
Minimum	15.3	13.1	272	218	53	53
Maximum	41.7	39.4	642	605	71	75
Sum	333	287	5605	4473	711	696
Count	12	11	12	11	12	11
Males	11½ W LF MALES	11½ W HF MALES	11½ W LF MALES	11½ W HF MALES	11½ W LF MALES	11½ W HF MALES
Mean	33.0	21.9	449	299	73	73
Standard Error	2.8	1.6	32	21	2.0	1.1
Median	30.3	22.9	440	309	71	72
Mode	#N/A	#N/A	#N/A	#N/A	71	71
Standard Deviation	9.8	5.7	112	74	6.9	3.8
Variance	95.4	32.2	12434	5402	47.2	14.4
Kurtosis	1.3	-0.5	-0.7	-0.8	0.1	0.1
Skewness	1.1	-0.5	0.2	-0.4	0.6	0.2
Range	35.2	18.2	356	223	24	14
Minimum	20.2	11.4	288	173	62	66
Maximum	55.4	29.6	644	396	86	80
Sum	396	263	5389	3584	878	876
Count	12	12	12	12	12	12

Table D.4 Descriptive statistics for the urinary aMT6s levels excreted in 24-h by gerbils aged 16 weeks.

Females	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
	16W LF FEMALES	16W HF FEMALES	16W LF FEMALES	16W HF FEMALES	16W LF FEMALES	16W HF FEMALES
Mean	29.7	24.2	442	346	67	68
Standard Error	2.4	3.3	36	42	1.6	2.9
Median	31.2	25.5	458	375	67	69
Mode	#N/A	#N/A	#N/A	#N/A	#N/A	69
Standard Deviation	8.2	11.6	126	147	5.5	9.9
Variance	66.7	134.4	15981	21604	30.4	99
Kurtosis	-0.2	-1.4	-0.2	-1.1	-1.0	2.8
Skewness	-0.8	-0.2	-0.4	-0.1	0.6	0.4
Range	25.4	33.1	421	457	16	42
Minimum	14.9	7.2	236	115	61	49
Maximum	40.2	40.2	657	572	77	91
Sum	357	290	5305	4151	810	815
Count	12	12	12	12	12	12
Males	16W LF MALES	16W HF MALES	16W LF MALES	16W HF MALES	16W LF MALES	16W HF MALES
Mean	34.1	31.6	397	399	85	78
Standard Error	4.2	3.1	42.7	32.3	2.7	1.7
Median	32.2	30.6	382	388	82	78
Mode	#N/A	#N/A	#N/A	#N/A	81.1	#N/A
Standard Deviation	14.5	10.9	148	112	9.5	6.1
Variance	209.4	118.8	21877	12487	89.4	36.6
Kurtosis	-1.1	2.0	-0.7	2.2	2.4	-0.5
Skewness	0.4	1.3	0.3	1.3	1.3	-0.2
Range	43.0	38.6	472	394	34	19
Minimum	13.4	19.3	181	278	73	68
Maximum	56.4	57.8	652	671	108	87
Sum	409	379	4766	4786	1014	938
Count	12	12	12	12	12	12

Table D.5 Descriptive statistics for the urinary aMT6s levels excreted in 24-h by male gerbils aged 9 weeks. It includes data from: i) the longitudinal study; ii) additional gerbils on 2 x 24-h urine collections.

MALES	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
	9W LF MALES	9W HF MALES	9W LF MALES	9W HF MALES	9W LF MALES	9W HF MALES
Mean	27.9	21.3	425	348	66	61
Standard Error	2.2	1.1	33	19	1.4	1.0
Median	25.8	20.2	410	346	65	62
Mode	#N/A	15.7	#N/A	#N/A	62	64
Standard Deviation	7.7	5.1	113	85	4.9	4.6
Variance	58.7	26.2	12778	7163	23.8	22
Kurtosis	-0.6	-0.4	-0.7	-0.4	-0.5	-0.7
Skewness	0.6	0.5	0.4	0.5	0.7	-0.2
Range	24.4	18.3	376	308	15	17
Minimum	18.4	13.3	252	213	60	53
Maximum	42.8	31.6	628	521	75	70
Sum	335	426	5099	6955	790	1227
Count	12	20	12	20	12	20

Table D.6 Descriptive statistics for the mean total urinary aMT6s levels excreted by all gerbils at 11½ weeks. It includes data from: i) the longitudinal study; ii) additional gerbils on 2 x 24-h urine collections.

	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
FEMALES	11½W LF FEMALES	11½W HF FEMALES	11½W LF FEMALES	11½W HF FEMALES	11½W LF FEMALES	11½W HF FEMALES
Mean	26.6	24.4	450	389	59	63
Standard Error	1.2	1.5	21	23	0.9	1.5
Median	26.2	24.0	424	369	58	63
Mode	26.3	#N/A	#N/A	#N/A	64	66
Standard Deviation	6.7	7.6	117	113	4.7	7.2
Variance	44.5	57.2	13701	12781	22.5	52
Kurtosis	0.4	-1.0	0.1	-0.7	-0.5	-0.5
Skewness	0.3	0.3	0.4	0.5	0.5	-0.2
Range	29.5	26.3	520	387	18	28
Minimum	12.2	13.1	205	218	53	48
Maximum	41.7	39.4	725	605	71	76
Sum	797	587	13505	9344	1779	1505
Count	30	24	30	24	30	24
MALES	11½W LF MALES	11½W HF MALES	11½W LF MALES	11½W HF MALES	11½W LF MALES	11½W HF MALES
Mean	33.0	21.7	449	310	73	70
Standard Error	2.8	1.4	32	19	2.0	1.2
Median	30.3	20.9	440	291	71	71
Mode	#N/A	#N/A	#N/A	#N/A	71	71
Standard Deviation	9.8	6.5	112	86	6.9	5.4
Variance	95.4	42.6	12434	7404	47.2	30
Kurtosis	1.3	-0.4	-0.7	0.5	0.1	-0.3
Skewness	1.1	0.5	0.2	0.7	0.6	-0.2
Range	35.2	24.0008	356	342	24	21
Minimum	20.2	11.4	288	173	62	59
Maximum	55.4	35.4008	644	515	86	80
Sum	396	456	5389	6508	878	1465
Count	12	21	12	21	12	21

Table D.7 Descriptive statistics for urinary aMT6s levels excreted by gerbils aged 16 weeks. It includes data from: i) longitudinal study; ii) additional gerbils on 2 x 24-hour urine collections.

	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
Females	16W LF FEMALES	16W HF FEMALES	16W LF FEMALES	16W HF FEMALES	16W LF FEMALES	16W HF FEMALES
Mean	25.2	23.5	380	340	66	68
Standard Error	2.2	2.6	33	34	1.4	2.3
Median	26.1	23.6	364	376	66	69
Mode	#N/A	#N/A	#N/A	#N/A	68	69
Standard Deviation	9.5	10.4	139	134	6.1	9.1
Variance	89.8	109.1	19228	18016	37.3	83
Kurtosis	-1.6	-1.1	-1.1	-1.0	0.2	2.5
Skewness	0.1	-0.1	0.3	0.0	-0.1	0.5
Range	26.9	33.0	468	457	24	42
Minimum	13.3	7.2	189	115	53	49
Maximum	40.2	40.2	657	572	77	91
Sum	454	377	6846	5437	1190	1086
Count	18	16	18	16	18	16
Males	16W LF MALES	16W HF MALES	16W LF MALES	16W HF MALES	16W LF MALES	16W HF MALES
Mean	28.1	30.4	342	393	80	77
Standard Error	2.9	2.0	29	21	2.0	1.4
Median	21.7	29.7	287	395	79	77
Mode	#N/A	#N/A	#N/A	388	81	71
Standard Deviation	13.1	8.8	132	94	9.0	6.4
Variance	172	78	17347	8900	82	41
Kurtosis	0.3	4.0	0.5	2.9	3.1	-0.8
Skewness	1.2	1.6	1.1	1.2	1.4	-0.1
Range	43.0	38.5	471	409	41	22
Minimum	13.4	19.3	181	263	67	65
Maximum	56.4	57.8	652	672	108	87
Sum	590	607	7184	7854	1688	1537
Count	21	20	21	20	21	20

Table D.8 Descriptive statistics for mean urinary aMT6s levels excreted by gerbils aged 28 weeks; over 48-hours in 2 x 24-hour intervals. The table includes 9 instances when the levels of aMT6s in day 1 are used.

	aMT6s ng/24-h		aMT6s pg/g BW/24-h		body weight g	
Females	28W LF FEMALES	28W HF FEMALES	28W LF FEMALES	28W HF FEMALES	28W LF FEMALES	28W HF FEMALES
Mean	17.4	21.7	248	289	70	76
Standard Error	1.2	1.6	15	23	1.2	1.7
Median	17.5	18.7	249	248	70	76
Mode	#N/A	#N/A	#N/A	#N/A	67.9	#N/A
Standard Deviation	4.4	5.7	58	83	4.4	6.2
Variance	19.4	32.5	3332	6909	18.9	38
Kurtosis	-0.7	-0.1	0.1	-0.6	0.5	1.6
Skewness	-0.2	1.0	-0.2	0.9	0.2	0.4
Range	14.8	17.6	217	238	17	25
Minimum	9.2	15.4	135	213	62	64
Maximum	23.9	33.0	353	450	79	89
Sum	244	282	3469	3759	983	983
Count	14	13	14	13	14	13
Males	28W LF MALES	28W HF MALES	28W LF MALES	28W HF MALES	28W LF MALES	28W HF MALES
Mean	24.4	27.8	273	306	95	91
Standard Error	1.6	2.6	34	30	6.0	2.5
Median	22.7	27.3	204	307	106	90
Mode	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
Standard Deviation	4.7	7.5	102	86	18.1	6.9
Variance	22.4	55.6	10407	7326	327.9	48
Kurtosis	-1.3	1.5	-2.2	-0.4	-2.2	-0.8
Skewness	0.3	-0.4	0.3	-0.3	-0.3	0.6
Range	13.3	25.7	247	265	42	20
Minimum	18.5	13.9	166	162	72	83
Maximum	31.9	39.6	413	427	114	103
Sum	220	222	2461	2448	857	729
Count	9	8	9	8	9	8

Table E.1 Results of areas of ventral glands in gerbils

9 week low-F males				
sample	length cm	breadth cm	area cm^2	body weight (g)
9W LF M1	2.45	0.60	1.16	67
9W LF M2	2.3	0.55	0.99	62
9W LF M3	2.0	0.70	1.10	63
9W LF M4	2.0	0.50	0.79	61
9W LF M5	1.8	0.55	0.78	58
9W LF M6	1.8	0.50	0.71	54
9W LF M7	2.55	0.55	1.10	67
		mean	0.95	61.7
		SD	0 2	4 7

9 week high-F males				
sample	length cm	breadth cm	area cm^2	body weight (g)
9W HF M1	2.4	0.4	0.75	62
9W HF M2	2.6	0.5	1.02	65
9W HF M3	2.3	0.5	0.90	53
9W HF M4	2.55	0.5	1.00	64
9W HF M5	2.15	0.6	0.98	55
9W HF M6	2.3	0.55	0.99	56
9W HF M7	2.4	0.7	1.32	68
9W HF M8	2.5	0 65	1.28	70
		mean	1.03	61.6
		SD	0.2	5.7

28 week low-F males				
28W LF M1	2.6	0.8	1.63	113
28W LF M2	2.7	0.7	1.48	108
28W LF M3	2.3	0.7	1.26	106
28W LF M4	2.4	0.6	1.13	114
28W LF M5	2.2	0.6	1.04	84
28W LF M6	2.2	0.6	1.04	77
28W LF M7	2.5	0.6	1.18	109
28W LF M8	1.9	0.5	0.75	72
		mean	1.19	98
		SD	0.3	17.2

28 week high-F males				
28W HF M1	2.4	0.6	1.13	83
28W HF M2	2.1	0.5	0.82	86
28W HF M3	2.65	0.6	1.25	97
28W HF M4	2.5	0.7	1.37	103
28W HF M5	2.3	0.55	0.99	85
28W HF M6	2.8	0.8	1.76	93
28W HF M7	2.65	0.7	1.46	95
28W HF M8	2.5	0.75	1.47	87
		mean	1.28	91
		SD	0.3	7 0

11½ week low-F females				
sample	length cm	breadth cm	area cm^2	body weight (g)
11½W LFF1	1.4	0.5	0.55	66
11½W LFF2	1.2	0.6	0.57	63
11½W LFF3	indistinguishable			65
11½W LFF4	indistinguishable			59
11½W LFF5	indistinguishable			55
11½W LFF6	indistinguishable			65
11½W LFF7	indistinguishable			61
11½W LFF8	indistinguishable			58
11½W LFF9	indistinguishable			56
11½W LFF10	indistinguishable			54
11½W LFF11	indistinguishable			53
11½W LFF12	indistinguishable			50
11½W LFF13	indistinguishable			50
11½W LFF14	indistinguishable			54
11½W LFF15	indistinguishable			51
11½W LFF16	indistinguishable			56
11½W LFF17	indistinguishable			56
11½W LFF18	indistinguishable			57
11½W LFF19	indistinguishable			60
11½W LFF20	indistinguishable			56
11½W LFF21	indistinguishable			54
11½W LFF22	indistinguishable			58
11½W LFF23	1.35	0.3	0.32	65
11½W LFF24	indistinguishable			54
11½W LFF25	0.9	0.3	0.21	59

11½ week high-F females				
sample	length cm	breadth cm	area cm^2	body weight (g)
11½WHF F1	indistinguishable			58
11½WHF F2	indistinguishable			55
11½WHF F3	1.15	0.45	0.41	63
11½WHF F4	1.6	0.5	0.63	76
11½WHF F5	1.2	0.35	0.33	63
11½WHF F6	1.3	0.45	0.46	67
11½WHF F7	1.3	0.5	0.51	71
11½WHF F8	1.25	0.35	0.34	70
11½WHF F9	1.45	0.4	0.46	68
11½WHF F10	indistinguishable			61

28 week low-F females				
28W LF F1	1.6	0.45	0.57	73
28W LF F2	1.6	0.45	0.57	71
28W LF F3	1.5	0.5	0.59	72
28W LF F4	1.3	0.45	0.46	70
28W LF F5	1.5	0.5	0.59	69
28W LF F6	1.9	0.45	0.67	72
28W LF F7	1.55	0.6	0.73	79
28W LF F8	1.5	0.45	0.53	75
		MEAN	0.59	72.6
		STDEV	0.08	3 2

28 week high-F females				
28W HF F1	1.60	0.50	0.63	73
28W HF F2	0.90	0.40	0.28	64
28W HF F3	1.70	0.50	0.67	78
28W HF F4	1.60	0.40	0.50	78
28W HF F5	1.75	0.55	0.76	74
28W HF F6	1.70	0.40	0.53	68
28W HF F7	1.60	0.50	0.63	72
28W HF F8	1.6	0.5	0.63	73
28W HF F9	1.5	0.45	0.53	78
28W HF F10	1.5	0.5	0.59	73
28W HF F11	1.6	0.5	0.63	78
28W HF F12	1.6	0.45	0.57	89
		MEAN	0.58	74.8
		STDEV	0.12	6.2

Table E.2 Results of the *t*-tests assuming unequal variances between the body weights of gerbils at 7, 9 and 11½ weeks

7 weeks of age	7W LF FEMALES	7W HF FEMALES	7W LF MALES	7W HF MALES
Mean	45	51	54	54
Variance	9.2	31.1	9.2	33.8
Observations	12	12	12	12
Hypothesized Mean Difference	0		0	
df	17		17	
t Stat	-3.226		0.044	
P(T<=t) one-tail	0.002		0.483	
t Critical one-tail	1.740		1.740	
P(T<=t) two-tail	0.005		1.0	
t Critical two-tail	2.110		2.110	
9 weeks of age	9W LF FEMALES	9W HF FEMALES	9W LF MALES	9W HF MALES
Mean	54	55	64	61
Variance	11.6	15.6	26.7	21.6
Observations	12	12	19	20
Hypothesized Mean Difference	0		0	
df	22		36	
t Stat	-0.769		1.851	
P(T<=t) one-tail	0.225		0.036	
t Critical one-tail	1.717		1.688	
P(T<=t) two-tail	0.5		0.1	
t Critical two-tail	2.074		2.028	
11½ weeks of age	11½W LF FEMALES	11½W HF FEMALES	11½W LF MALES	11½W HF MALES
Mean	58	62	70	70
Variance	27.5	54.8	59.8	29.7
Observations	39	25	18	21
Hypothesized Mean Difference	0		0	
df	39		30	
t Stat	-2.308		-0.026	
P(T<=t) one-tail	0.013		0.490	
t Critical one-tail	1.685		1.697	
P(T<=t) two-tail	0.03		1.0	
t Critical two-tail	2.023		2.042	

Table E.3 Results of the *t*-tests assuming unequal variances between the body weights of gerbils at 16 and 28 weeks

16 weeks of age	16W LF FEMALES	16W HF FEMALES	16W LF MALES	16W HF MALES
Mean	64	68	78	77
Variance	46.5	83.5	72.4	32.3
Observations	27	16	32	18
Hypothesized Mean Difference	0		0	
df	25		46	
t Stat	-1.499		0.656	
P(T<=t) one-tail	0.073		0.258	
t Critical one-tail	1.708		1.679	
P(T<=t) two-tail	0.146		0.5	
t Critical two-tail	2.060		2.013	
28 weeks of age	28W LF FEMALES	28W HF FEMALES	28W LF MALES	28W HF MALES
Mean	70	76	95	91
Variance	18.9	37.9	327.9	48.3
Observations	14	13	9	8
Hypothesized Mean Difference	0		0	
df	21		11	
t Stat	-2.631		0.626	
P(T<=t) one-tail	0.008		0.272	
t Critical one-tail	1.721		1.796	
P(T<=t) two-tail	0.02		0.5	
t Critical two-tail	2.080		2.201	

Table E.4 Combined testes weights in gerbils at 16 weeks

16 week	testes weight (g)			body weight	relative testes wt	16 week	testes weight (g)			body weight	relative testes wt
low-F	RHS	LHS	sum	g	g/100 g BW	high-F	RHS	LHS	sum	g	g/100 g BW
M1	0.65	0.76	1.41	88	1.61	M1	0.48	0.60	1.08	78	1.38
M2	0.68	0.66	1.34	82	1.64	M2	0.59	0.53	1.12	86	1.30
M3	0.76	0.76	1.52	88	1.73	M3	0.55	0.64	1.19	75	1.59
M4	0.65	0.65	1.30	82	1.59	M4	0.56	0.56	1.12	69	1.62
M5	0.51	0.73	1.24	81	1.53	M5	0.62	0.59	1.21	78	1.55
M6	0.46	0.50	0.96	74	1.29	M6	0.45	0.43	0.88	68	1.29
M7	0.80	0.82	1.62	108	1.50	M7	0.51	0.56	1.07	76	1.41
M8	0.59	0.66	1.25	91	1.37	M8	0.65	0.67	1.32	86	1.54
M9	0.57	0.65	1.22	81	1.50	M9	0.49	0.58	1.07	80	1.34
M10	0.77	0.78	1.55	91	1.71	M10	0.52	0.53	1.05	87	1.21
M11	0.65	0.65	1.30	76	1.71	M11	0.57	0.54	1.11	76	1.46
M12	0.58	0.58	1.16	73	1.58	M12	0.49	0.49	0.98	80	1.23

	Testes weights		Relative testes weights	
t-Test: Two-Sample Assuming Unequal Variances	16W LF	16W HF	16W LF	16W HF
Mean	1.32	1.10	1.56	1.41
Variance	0.03	0.01	0.02	0.02
Observations	12	12	12	12
Hypothesized Mean Difference	0		0	
df	18		22	
t Stat	3.595		2.715	
P(T<=t) one-tail	0.001		0.006	
t Critical one-tail	1.734		1.717	
P(T<=t) two-tail	0.002		0.01	
t Critical two-tail	2.101		2.074	

Descriptive statistics	Testes weights		Relative testes weights	
	16W LF	16W HF	16W LF	16W HF
Mean	1.32	1.10	1.56	1.41
SEM	0.05	0.03	0.04	0.04
Median	1.30	1.10	1.58	1.40
Mode	1.30	1.12	#N/A	#N/A
SD	0.18	0.11	0.14	0.14
Variance	0.03	0.01	0.02	0.02
Kurtosis	0.2	1.2	0.10	-1.36
Skewness	-0.1	0.0	-0.74	0.09
Range	0.7	0.4	0.44	0.42
Minimum	0.96	0.88	1.29	1.21
Maximum	1.62	1.32	1.73	1.62
Sum	15.87	13.20	18.77	16.92
Count	12	12	12	12

Shaded cells represent undescended testes.

Table F.1 Results of antiserum dilution curves: standard curves using different dilutions of antiserum

Standard aMT6s ng/ml	1:10 000			1:20 000			1:30 000			1:40 000		
	cpm	bound	B/T%	cpm	bound	B/T%	cpm	bound	B/T%	cpm	bound	B/T%
T	7734			7390			7473			7718		
NSB	7558			6668			7322			6877		
0	900	6834	88.4	893	6497	87.9	1185	6288	84.1	1612	6106	79.1
0.5	958	6776	87.6	1047	6343	85.8	1454	6019	80.5	2232	5486	71.1
1	991	6743	87.2	1217	6173	83.5	1762	5711	76.4	2910	4808	62.3
2	1082	6652	86.0	1585	5805	78.6	2372	5101	68.3	3820	3898	50.5
4	1463	6271	81.1	2302	5088	68.8	3310	4163	55.7	4688	3030	39.3
7	2192	5542	71.7	3336	4054	54.9	4342	3131	41.9	5677	2041	26.4
10	2452	5282	68.3	3959	3431	46.4	4692	2781	37.2	6070	1648	21.4
20	3737	3997	51.7	5410	1980	26.8	5633	1840	24.6	7058	660	8.6
50	5141	2593	33.5	6346	1044	14.1	6214	1259	16.8	7440	278	3.6

Table F.2 (i) Limit of detection for RIAs for urinary aMT6s

assay no	cpm	mean cpm	SD	SEM	CI	mean cpm at 0.5 pg standard	mean cpm at 1 pg standard	m	c	limit detection
1	2492 2387 2387 2422	2422	49.5	24.7	2501	3112	3774	1324.0	2450	0.04
2	2986 3204 2954 2933	3019	125	62.5	3218	3576	4168	1184.0	2984	0.20
3	2899 2705 2840 2737	2795	90	45.0	2938	3534	4146	1224.0	2922	0.01
4	2840 2729 2725 2823	2779	61	30.4	2876	3373	3860	974.0	2886	-0.01
5	2634 2381 2742 2670	2607	157	78.5	2857	3434	4129	1390.0	2739	0.08
						1pg std	2pg std			
6	2883 2658 2524 2716	2695	149	74.4	2932	3987	4646	659.0	3328	-0.60
7	397 374 404 401	394	13.6	6.8	416	604	843.0	478.0	365	0.11
8	437 363 387 353	385	37.5	18.7	445	614	823.0	418.0	405	0.09
9	3627 3629 3569 3724	3637	64.2	32.1	3739	4008	4340	664.0	3676	0.10
10	3026 2947 2920 2938	2958	47	23.4	3032	3636	4007	742.0	3265	-0.31
							2 pg			
12	3014 3222 3164 3139	3135	87.7	43.8	3274	3492	4449	957.0	2535	0.77

Table F.2 (ii) Limit of detection for RIAs for urinary aMT6s

assay no	cpm	mean cpm	SD	SEM	CI	mean cpm at 0.5 pg standard	mean cpm at 1 pg standard	m	c	limit detection
						2pg	4pg			
13	4133 4069 4221 4198	4155	69	34.3	4264	5249	5983	367.0	4515	-0.68
14	2825 2873 2866 2837	2850	22.9	11.5	2887	3415	3987	1144.0	2843	0.04
15	3278 3364 3252 3370	3316	60	29.9	3411	3693	4005	624.0	3381	0.05
16	2932 2998 2945 2973	2962	29.5	14.7	3009	3559	4080	1042.0	3038	-0.03
17	3312 3600 3319 3183	3354	175.8	87.9	3633	3494	3939	890.0	3049	0.66
18	3370 3125 3329 3204	3257	113	56.4	3436	3626	3990	728.0	3262	0.24
19	4007 3762 3769 3820	3840	115	57.3	4022	4151	4382	462.0	3920	0.22
20	2560 2565 2533 2493	2538	33	16.5	2590	3200	3839	1278.0	2561	0.02
21	2940 3086 3111 3181	3080	101	50.7	3241	3579	3928	698.0	3230	0.02
22	2962 2813 2581 2710	2767	161	80.6	3023	3265	3929	1328.0	2601	0.32
23	3404 3296 3800 3483	3496	217	108.4	3841	3800	4145	690.0	3455	0.56
24	2980 2809 2877 2841	2877	74	37.1	2995	3603	4445	1684.0	2761	0.14

Table F.2 (iii) Limit of detection for RIAs for urinary aMT6s

assay no	cpm	mean cpm	SD	SEM	CI	mean cpm at 0.5 pg standard	mean cpm at 1 pg standard	m	c	limit detection
25	2872 2776 2801 2813	2816	41	20.3	2880	3499	4190	1382.0	2808	0.05
26	2724 2601 2734 2782	2710	77	38.6	2833	3544	4177	1266.0	2911	-0.06
27	2467 2372 2445 2453	2434	42.5	21.2	2502	3328	3909	1162.0	2747	-0.21
28	2398 2446 2498 2331	2418.3	71	35.5	2531	2766.5	2945	357.0	2588	-0.16
29	2563 2322 2439 2485	2452	101	58.2	2637	2867	3017	300.0	2717	-0.27
30	3244 3355 3273 3385	3314	67	33.3	3420	3952	4265	626.0	3639	-0.35
31	3232 3229 3288 3322	3268	45	22.6	3340	3948	4357	818.0	3539	-0.24
32	3131 3188 3031 3026	3094	79	39.6	3220	3650	4052	804.0	3248	-0.03
						1pg	2pg			
33	3109 2840 2949 2906	2951	114	57.2	3133	4079	4850	771.0	3308.0	-0.23
								mean		**0.02**

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