The Parkes Lecture* Heat and the testis

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The evidence for the lower temperature of the testes of many mammals is summarized, and the reasons suggested for the descent of the testes into a scrotum are discussed. Descriptions are given of the various techniques used for studying the effects of heat on the testis, whole body heating, local heating of the testes (by inducing cryptorchidism, scrotal insulation or immersion of the scrotum in a water bath), and heating of tissue or cell preparations *in vitro*. The effects of heat are discussed, effects on the testis (weight, histology, physiology, biochemistry and endocrinology), on the numbers and motility of spermatozoa in rete testis fluid and semen, on fertilizing ability of spermatozoa and on the subsequent development of the embryos produced when spermatozoa from heated testes are used to fertilize normal ova. The possible mechanisms for the damaging effects of heat are discussed, as well as the importance of heat-induced abnormalities in male reproduction in domestic animals and humans.

Introduction

All tissues are susceptible to damage by heat. However, the testes are unusual in that they are damaged by exposure to the temperatures normally found within the abdomen, although they originate within the abdominal cavity and in many mammals descend into a scrotum only during fetal or neonatal development. In some mammals, and in all birds and all other classes of animal, the testes remain within the abdomen, and body temperature is considerably higher in birds than in mammals. There was a suggestion that the testes of birds are cooler than the other abdominal organs because of the proximity of some of the airsacs, but this is no longer believed to be the case (Beaupré *et al.*, 1997). There is abundant evidence that the scrotal testes of mammals in a thermoneutral environment are appreciably cooler than the body (see Setchell, 1978; Waites and Setchell, 1990), but this does not mean that they are kept at a constant lower temperature. Because of the countercurrent heat exchange function of the blood vessels in the spermatic cord, the testes are kept at a uniform temperature throughout, and at approximately the same temperature as the subcutaneous scrotal tissue (see Setchell *et al.*, 1994a; Setchell and Mieusset, 1996). If the animal is placed in a hot environment and evaporation of sweat from the scrotal skin cannot keep the scrotal skin cool, then the testis warms up (see Setchell and Mieusset, 1996). There is an approximately linear relationship between air temperature and subcutaneous skin temperature in the scrota of rams exposed to field conditions in an Australian summer (Setchell *et al.*, 1994b), and when air temperature exceeds 40°C, scrotal

temperature rises to values normally found in the abdomen, while rectal temperature shows only a small rise. In rats acclimatized to an environment of 35°C, the intrascrotal temperature was similar to the deep body temperature of control animals, although the scrotal–body temperature difference was maintained (Sod-Moriah *et al.*, 1974).

If a testis does not descend normally into the scrotum, it is described as cryptorchid and bilaterally cryptorchid individuals are invariably sterile, although enough androgens are secreted to ensure that they have male characteristics and behaviour. Spontaneous cryptorchidism is reasonably common in humans (Hutson *et al.*, 1997), pigs and horses, but much less frequent in ruminants (Wensing and Colenbrander, 1986). It was suggested by Crew (1922) that the cryptorchid testis was 'aspermatic' because it was at a higher temperature. Subsequent studies showed that if a cryptorchid pig testis is artificially cooled, spermatogenesis resumes (Frankenhuis and Wensing, 1979). Likewise, spermatogenesis does not commence in the testes of rats made cryptorchid by cutting the gubernaculum at birth (Bergh and Damber, 1978). If the testes are returned to the abdomen at 17 days of age, spermatogenesis does not begin until the testis is returned to the scrotum (Karpe *et al.*, 1981, 1984), and the longer the time that elapses before this is done, the greater is the long-term effect. Elephants and hyraxes normally retain their testes close to the kidneys, and testis temperature in these animals is similar to body temperature (see Setchell and Mieusset, 1996), without apparently impairing their ability to produce spermatozoa. In one species of tropical rodent, *Zygodontomys brevicauda*, the testes continue to produce spermatozoa when the testes are returned to the abdomen, even though there is a small reduction in testis weight (Bronson and Heideman, 1993).

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However, alternative means of cooling abdominal testes appear to have evolved in Cetaceae, in which cool venous blood from the tail and dorsal fin is carried into the abdominal cavity in veins that lie interspersed among the arteries running to the testes (Rommel *et al.*, 1992). Although the full physiological significance of this arrangement has not been determined, it does appear that tissues in the vicinity of these veins are at a lower temperature than other abdominal organs (Rommel *et al.*, 1994; Pabst *et al.*, 1995). In seals, the testes are outside the body cavity, but under a thick layer of blubber. Nevertheless, they too are considerably cooler than the abdominal cavity (Bryden, 1967; Blix *et al.*, 1983), again because venous blood from the extremities is carried close to the testes and their arterial supply (Rommel *et al.*, 1995). Therefore, although a few mammals appear to be able to produce spermatozoa in testes at body temperature, evolution does appear to have taken several routes to cooling the testes in most members of this class.

It is still a mystery why the testes of most mammals appear to need a lower temperature for normal function. Various suggestions have been made. It was thought (Ehrenberg *et al.*, 1957) that the lower temperature might have the effect of reducing the higher mutation rate in males, but this appears to be due to a substantially lower mutation rate in the genes on the X chromosome than on the autosomes, and because there are so many more genes on the X chromosome than on the Y, this lowers the mean rate for the XX females (McVean and Hurst, 1997). Furthermore, it has recently been suggested that the higher mutation rate in males is important as a source of genetic novelty and it has been shown that there is also a higher mutation rate in genes on the Z chromosome than in those on the W chromosome in birds. Male birds are ZZ, and the higher mutation rate in males is therefore more likely to be due to the greater number of cell divisions leading to the formation of spermatozoa than eggs (Ellegren and Fridolfsson, 1997). The lower temperature of the testis would presumably lead to a lower metabolic rate, although it is not clear why this should be an advantage. Various other suggestions for the evolution of the scrotum have been made, including that the scrotum developed as a form of sexual decoration (Portmann, 1952, but cf Ruibal, 1957; Cowles, 1965), that testicular descent decreases blood supply to maturing germ cells, so that the mitochondria enhance their enzymatic machinery for oxidative metabolism, resulting in increased aerobic fitness which is an advantage in interejaculate competition (Freeman, 1990) and even that the testes migrate out of the abdominal cavity in species that leap around most (Chance, 1996).

Techniques for studying the effects of heat on the testis

Whole body heating

In one sense the most physiological way of studying the effects of heat on the testis is to expose the whole animal to a hot environment. However, this involves two complicating factors. First, the body reacts to heat stress in a variety of ways, and there are significant physiological, metabolic and

endocrinological changes that could affect the testis indirectly. Second, the ability of the scrotal skin to produce sweat can be influenced by the prior exposure of the animal to heat, and therefore for a given heat exposure, the temperature to which the testis is exposed may be quite different. Rams studied at the end of summer have much greater capacity for scrotal sweat production and hence testis cooling, than the same animals in winter (Waites and Voglmayr, 1963), and rams of two different strains, which had much different sensitivities to whole body heating, showed the same changes in semen characteristics and fertility when their testes were exposed directly to the same heat load (Fowler, 1968). Testes of rats kept at 35°C from weaning to 100 days old or for at least 30 days were no smaller than those of controls kept at 22 or 21°C, although body weight and the weights of many other organs were reduced (Ray *et al.*, 1968; Magal *et al.*, 1981). Similarly, in a strain of mice that had been bred for many generations at 33°C, a temperature normally causing appreciable infertility in this species unless they are exposed from weaning (Pennycuik, 1967), the testes were no smaller than those of animals of a similar genetic background kept at 21°C, and were no more resistant to local heating of the testes than were those of control mice (van Zelst *et al.*, 1995). This finding suggests that the heat-adapted animals had simply developed more efficient mechanisms for cooling their testes.

Local heating of the testes

Local heating of the testes has usually been achieved in one of three ways, induced cryptorchidism, scrotal insulation or short-term heating, usually by immersion in a water bath. Cryptorchidism can be produced easily in adult rodents (Piana and Savarese, 1991; Nelson, 1951; Clegg, 1960, 1963; Niemi and Korman, 1965; Davis and Firlit, 1966; Meistrich *et al.*, 1973; Risbridger *et al.*, 1981; Sharpe, 1983; Fujisawa *et al.*, 1988; Yin *et al.*, 1997) and rabbits (Fukui, 1923b; Plöen, 1972, 1973a,b), because the inguinal canal remains patent and sufficiently large for the testis to be pushed up into the abdominal cavity. The testis must be held there, either by suturing it to the abdominal wall, or by closing the inguinal canal and in many cases the gubernaculum is cut. This technique exposes the testis to abdominal temperature, and the testis can be returned to the scrotum after a suitable interval. However, in many studies, the testis is left in the abdominal cavity until the selected observations are made. Cryptorchidism has also been produced in dogs (Griffiths, 1893), sheep (Moore and Oslund, 1924; Skinner and Rowson, 1968; Hochereau-de Reviers *et al.*, 1979; Barenton *et al.*, 1982; Lunstra and Schanbacher, 1988) and wallabies (Setchell and Thorburn, 1970), although in these animals the inguinal canal though patent is not large enough for the testis to pass through without surgical intervention. By making only one testis cryptorchid, the other testis of the same animal can be used as a control, and this also minimizes, but does not always eliminate, changes in peripheral hormone concentrations. Cryptorchidism can also be induced in newborn rats by cutting the gubernaculum (Bergh *et al.*, 1978), or in immature

animals before the testes have descended (Karpe *et al.*, 1981, 1984). Cryptorchidism can be induced by treating pregnant females with the anti-androgen, flutamide, although not all male offspring are affected (Husmann and McPhaul, 1991; Spencer *et al.*, 1991; van der Schoot, 1992) or with oestrogens (Rajfer and Walsh, 1977; Rajfer, 1982), although this treatment is more effective in mice than in rats (cf Hutson *et al.*, 1990 and Boylan, 1978). The temperature of the testis can also be raised towards body temperature in sheep by shortening the scrotum, thereby pushing the testes up against the body wall (Thwaites *et al.*, 1982; Sanford *et al.*, 1988).

The testes can also be heated by insulating the scrotum, a technique that is very convenient in rams (Glover, 1955, 1956; Byers and Glover, 1984; Mieusset *et al.*, 1992a), bulls (Ross and Entwistle, 1979; Prabhakar *et al.*, 1990; Sidibe *et al.*, 1992; Karabinus *et al.*, 1997), stallions (Freidman *et al.*, 1991) and boars (Malmgren, 1989, 1990), and the insulation can be continuous or intermittent, for example 8, 12 or 16 h per 24 h (Mieusset *et al.*, 1991, 1992a); insulation for only 8 h per day can eventually produce effects on spermatogenesis. Effects can even be produced by insulating only the neck of the scrotum in bulls (Kastelic *et al.*, 1996). Humans can be trained to raise their testis temperature by 2–3° for up to 30 min each day by biofeedback training (French *et al.*, 1973), and testis temperature can also be raised by holding the testes against the perineal region with specially designed underclothing (Rock and Robinson, 1965; Robinson and Rock, 1967; Rock *et al.*, 1968; Mieusset *et al.*, 1985, 1987a,b). Heat can also be applied by lamps, microwave or ultrasound (Fukui, 1923a, 1923–4; Imig *et al.*, 1948; Fahim *et al.*, 1975; Dumontier *et al.*, 1977).

However, the most precise way of exposing a testis to an increased temperature for a short time is to immerse the scrotum in a water bath at the chosen temperature, usually with the animals anaesthetized or sedated. This technique has been used in rats (Steinberger and Dixon, 1959; Collins and Lacy, 1969; Chowdhury and Steinberger, 1964, 1970; Main *et al.*, 1978; Jegou *et al.*, 1984; Galil and Setchell, 1988a,b; Setchell *et al.*, 1996), guinea-pigs (Moore, 1924a,b; Young, 1927), mice (Hand *et al.*, 1979; Reid *et al.*, 1981; Marigold *et al.*, 1985; De Vita *et al.*, 1990; Gasinska and Hill, 1990; van Zelst *et al.*, 1995; Sailer *et al.*, 1997; Setchell *et al.*, 1998) and rams (Setchell *et al.*, 1991; Mieusset *et al.*, 1992b). By pushing one testis back into the abdomen and retaining it there during the heating, unilateral effects can be studied (Galil and Setchell, 1988b). In rams, the scrotum can be enclosed in a small chamber, through which heated air can be circulated while heated water is pumped through tubing in the walls (Waites and Setchell, 1964; Fowler, 1968).

Heating in vitro

A few studies have examined the effects of heating the testis or testicular cells *in vitro*. Some have used isolated perfused testes (VanDemark and Ewing, 1963; Linzell and Setchell, 1969), whereas others have studied the effects of increased temperatures on incubated decapsulated testes (Biggiogera *et al.*, 1996), segments of seminiferous tubules

(Parvinen *et al.*, 1992; Cataldo *et al.*, 1997) or on isolated testicular cells or a Sertoli cell line (Zakeri *et al.*, 1990; Clark and Griswold, 1997).

Actions of heat on the testes

The actions of heat on the testis can be direct on specific cell types within the testis, but there are also indirect effects, resulting from the cellular changes occurring in the testis caused by the heating. While the latter are important in considering the effects of germ cells on the Sertoli and Leydig cells, in this review, attention will be mainly directed to the direct effects of heat. These effects can be demonstrated at five levels, effects on the testis itself, effects on the numbers, structure or motility of spermatozoa leaving the testis in rete testis fluid or those in semen, the fertilizing ability of those spermatozoa even when apparently normal in appearance and motility, and finally the development of the embryos resulting from *in vivo* or *in vitro* fertilization with spermatozoa from heat-treated males in normal females.

Effects on testis

Weight. One of the more obvious effects of acute or chronic exposure of the testes to heat is a reduction in testis weight. This occurs between 1 and 2 days after a single exposure of the testes of rats, and testis weight has reached a minimum of about 40% of control between 7 and 28 days after treatment at either 41°C for 60 min or 43°C for 30 min. Thereafter there is a return towards normal weight, but testis weight can still be below the control value for at least 63 days after a single heating (Setchell and Waites, 1972; Fridd *et al.*, 1975; Main *et al.*, 1978; Bartlett and Sharpe, 1987; Galil and Setchell, 1988b; McLaren *et al.*, 1994; Setchell *et al.*, 1996). Exposure of the testes of rats 7 times at 6 week intervals to 43.5°C for 20 min produced a decrease in testis weight of about 40%, which was maintained for at least 24 weeks after the last exposure (Bowler, 1972). If a rat is made cryptorchid, testis weight does not change in the first 24 h (Setchell, 1970 and unpublished results; Bergh and Damber, 1984), but has fallen below control values by the second day in young rats (Shikone *et al.*, 1994) and by 5–7 days in adult rats (Clegg, 1963; Fujisawa *et al.*, 1988). Exposure of unacclimatized mice to an environment of 35°C leads to a fall in testis weight within 4 days (Meistrich *et al.*, 1973). The reduction in testis weight after local heating is comparable in extent to that in rats (Hand *et al.*, 1979; Marigold *et al.*, 1985; Gasinska and Hill, 1990; Van Zelst *et al.*, 1995; Jannes *et al.*, 1998); after exposure to 42°C for 30 min, there is no change after 1 day, but a fall of almost 50% after 3 days (Sailer *et al.*, 1997). However, the decrease in testis weight after cryptorchidism was slower than that in rats, with significant difference of about 25% from control only becoming evident after 7 days (Meistrich *et al.*, 1973; Yin *et al.*, 1998). In rams, a decrease of about 50% in testis weight occurred 21 days after the testes had been exposed to 42°C for 45 min (Setchell *et al.*, 1991; Hochereau-de Reviers, 1993) and exposure of rams to a hot environment for 14 days caused a reduction in testis weight to about 70%

of control values (Gomes *et al.*, 1971). Cryptorchid testes weigh less than 20% of normal testes (Hochereau-de Reviers, 1979; Barenton *et al.*, 1982; Lunstra and Schanbacher, 1988) and testes from short-scrotum animals are also much smaller than those of normal animals (Sanford *et al.*, 1993).

Histology. Histological changes were observed in heated and cryptorchid testes by many early investigators (Moore, 1924a,b; Fukui, 1923a, 1923/4; Moore and Oslund, 1924), and changes in spermatocytes could be detected on the day after exposure to heat (Young, 1927). Later studies showed that increased numbers of abnormal pachytene spermatocytes could be detected as early as 1 h after the testes of rats had been exposed to 43°C for 15 min, although at that time there were no obvious effects on the number of spermatogonia (Chowdhury and Steinberger, 1964, 1970). Dye chromophilia could also be seen in pachytene and diplotene spermatocytes as early as 1 h after heating the testes to 42°C for 30 min, and by 2 h, there were changes in acid phosphatase and aminopeptidase in these cells (Blackshaw and Hamilton, 1970). In rats acclimatized for 3 months to an environment of 35°C, spermatogenesis was severely affected in about 20% of tubular cross-sections, whereas the remainder appeared normal (Sod-Moriah *et al.*, 1974). In rams, greater numbers of degenerating pachytene spermatocytes were found 12 h after a 150 min exposure of the testes to 40°C (Waites and Ortavant, 1968). There is one report that in pigs exposed to a hot environment for 90 days, the spermatids, but not pachytene spermatocytes, are reduced in number (Wettemann and Desjardins, 1979), but a later study using scrotal insulation for 100 h showed that both pachytene spermatocytes and early spermatids were affected and reduced in number by the end of the insulation period (Malmgren and Larsson, 1989). The sensitivity of the pachytene spermatocytes was also found in mice, as judged from the numbers of elongated spermatid nuclei, which could be counted in homogenates of testes, taken 14 days after heating for 30 min at 43°C (Gasinska and Hill, 1990). This result was confirmed using flow cytometry to assess the numbers of haploid cells (De Vita *et al.*, 1990), and although there was no change after 1 day, a fall in the percentage of haploid cells could be detected 3 days after exposure to 42°C for 60 min (Sailer *et al.*, 1997). The numbers of spermatids and spermatozoa in fragments of human testes were lower when they were cultured at 37°C, rather than 31°C, whereas the numbers of spermatogonia and spermatocytes were not affected (Nakamura *et al.*, 1987). Pachytene spermatocytes and young spermatids also appear to be the first cells to be affected in experimental cryptorchidism in rats (Davis and Firlit, 1966; Parvinen, 1973; Blackshaw and Massey, 1978) and rabbits, in which degenerating cells were found after 24 h (Plöen, 1973a); however, there were also changes at this time in spermatids (Plöen, 1972), and these became more pronounced later, even though the testis had been returned to the scrotum (Plöen, 1973b). In immature (Shikone *et al.*, 1994) and in mature rats (Henriksen *et al.*, 1995) and mature mice (Yin *et al.*, 1997), cryptorchidism induces increased apoptosis of germ cells, which in the adult animals mainly involved spermatocytes and round spermatids. However, there is one report that local heating of the testes appears to

cause increased apoptosis of spermatogonia (Allan *et al.*, 1987). Subsequent studies by Swerdloff *et al.* (1998) do not support this, and in their studies, the increased apoptosis seems to involve mostly spermatocytes (Lue *et al.*, 1998), but at a different stage of the cycle from that seen after gonadotrophin withdrawal (Sinha Hikim *et al.*, 1995, 1997).

However, even though it is now generally agreed that the pachytene spermatocytes and spermatids are the cells most easily affected, if the degree of heat exposure is increased, either by increasing the temperature or the time of exposure, then there are also effects on other cell types. Collins and Lacy (1969), in a study involving a range of times of exposure of rat testes to 43°C, suggested that there were two 'critical periods', beyond which cells in heated testes could not proceed. As the time of exposure was increased, a third critical period was revealed, and eventually, all these periods merged into one. Other authors (Idänpään-Heikkilä, 1966) reported effects on Sertoli cells and the basement membrane of the seminiferous tubules. In rams, as well as the effects on spermatocytes, there was also an increase in the numbers of dividing B-spermatogonia, which subsequently die (Waites and Ortavant, 1968). Counts of spermatid nuclei (Gasinska and Hill, 1990) or determining the percentage of haploid cells by flow cytometry (De Vita *et al.*, 1990; Sailer *et al.*, 1997) after 28 days indicated that the differentiating spermatogonia are less sensitive than the pachytene spermatocytes, but are still affected by the heating. The failure of the testis weight to return completely to control values even after more than 60 days, or after 24 weeks after repeated heating, which has already been mentioned, also suggests that the spermatogonia are not unaffected and Reid *et al.* (1981) showed that heating of the testis exacerbated the effects of radiation on the spermatogonia, as judged by repopulation of tubules 35 days after treatment.

It is also generally assumed that the timing of spermatogenesis is constant for any given species, but there is one study that suggests that the duration of the spermatogenic cycle in mice is decreased slightly in cryptorchid testes, and in animals kept at 32°C (Meistrich *et al.*, 1973). Incubating segments of seminiferous tubules at 37°C rather than at 32°C appeared to speed up the transition from one stage of spermatogenesis to the next (Parvinen *et al.*, 1992).

It has also been reported that there was an increased incidence of X-Y dissociation in primary spermatocytes in diakinesis-metaphase after exposure of mice to an environment of 35°C for 2, 3 or 5 days, an effect which reached a peak 5 days after the end of the heat stress; this would indicate that the primary effect was again on spermatocytes at the pachytene stage (Garriott and Chisman, 1980; Waldbieser and Chisman, 1986). The increased X-Y dissociation after exposure of cool-adapted mice to an environment of 33°C was subsequently confirmed, and it was also shown that the same effect could be produced by local heating of the testes of mice and rats (Van Zelst *et al.*, 1995).

Physiology. Most experiments, using a variety of techniques for measuring either total or capillary blood flow through the testis, indicate that blood flow through the testis

does not increase until testis temperature reaches values well above body temperature (Waites and Setchell, 1964; Setchell *et al.*, 1966; Waites *et al.*, 1973; Godinho and Setchell, 1975; Damber and Janson, 1978; Setchell *et al.*, 1995), except in marsupials (Setchell and Thorburn, 1969) although there is one study in anaesthetized rams which suggests that total blood flow does increase as the temperature is raised from the normal scrotal temperature of 33°C to 36°C, but there is no further increase as the temperature was raised to 39°C (Mieusset *et al.*, 1992b). In another experiment in rams exposed to an environment of 32°C for 7 days, blood flow through the testis showed an initial increase after 24 h, followed by a return to normal flow at 3 days, and then reduced to about half the normal blood flow; similar results were found when the testes were heated by enclosing the scrotum in an electric heating pad which raised the temperature of the testis to about 39°C (Dutt *et al.*, 1977). The failure to detect consistently any changes in flow when the temperature of the testis is increased contrast with studies in other tissues, where flow is very sensitive to increases in temperature, and in experiments where blood flow was measured at various times after exposure of the testes to heat. Under these circumstances, as the weight of the testis decreased, the blood flow per testis decreased in proportion, that is to say blood flow per unit weight of testis remained about the same (Galil and Setchell, 1988b; Setchell *et al.*, 1991).

However, one obvious change that is seen involves the pattern of blood flow as the temperature of the testis is changed. Vasomotion, the rhythmical fluctuations in flow with a frequency of about 10 per min unrelated to heart or respiration rate decreased in amplitude and increased in frequency as the temperature was raised, and finally disappeared; as the temperature was reduced again, vasomotion reappeared, amplitude increased and frequency decreased, until at temperatures below normal, there were very slow (about 6 per min) fluctuations with an amplitude of as much as 50% of average flow (Setchell *et al.*, 1995). The significance of these changes, or indeed the importance of vasomotion in the normal function of the testis, are not understood. However, it has been suggested that the fluctuations in flow in individual capillaries associated with vasomotion might be involved in determining rates of fluid filtration and reabsorption in tissues, and therefore it is a pity that no measurements appear to have been made on vascular permeability in the testis at various temperatures to either small or large molecules.

The blood–testis barrier, which limits the penetration of many substances into the seminiferous tubules, is only marginally affected by temperature. Flow of fluid from the rete testis, which depends on the integrity of the barrier, is reduced during heating in rams (Linzell and Setchell, 1969; Setchell *et al.*, 1971) but not in rats (Main and Waites, 1977), and the composition of the fluid was unaffected at the time of heating or subsequently (see also Setchell *et al.*, 1996). However, the entry rates for K⁺, Na⁺, lysine and some of the more slowly penetrating steroids (not testosterone) were increased during heating, although the entry of albumin was unaffected (Main and Waites, 1977). In cryptorchid rat testes, lanthanum salts were excluded from the tubules, just as they were in normal adult testes, while penetration to the lumen

was found in testes from prepubertal rats (Hagenäs *et al.*, 1977). However, some slight deterioration of the barrier 7 days after the testes were made cryptorchid could be demonstrated by measuring the rate of entry of radioactive inulin into luminal fluid collected from the seminiferous tubules by micropuncture (Turner *et al.*, 1982).

Biochemistry. The uptake of oxygen *in vivo* is significantly increased during heating of the testes of rams, but with little change in the uptake of glucose or the production of lactate (Waites and Setchell, 1964; Free and VanDemark, 1968). Pieces of seminiferous tubule incubated *in vitro* secreted less lactate than do tubules from scrotal testes (Bergh *et al.*, 1987). The rat testis has been shown to contain the glucose transporters GLUT 2 and 3 in appreciable amounts, and GLUT 2 is increased and GLUT 3 reduced in testes that had been cryptorchid for 10, 20 or 30 days (Farooqui *et al.*, 1997). However, these studies make no reference to other reports which showed that the endothelial cells of the testis contain GLUT 1, the glucose transporter usually associated with the blood–brain barrier (Holash *et al.*, 1993).

The incorporation of labelled amino acids into protein is decreased when isolated spermatids are incubated at 38°C instead of 34°C (Nakamura *et al.*, 1978; Nakamura and Hall, 1980). When seminiferous tubules were isolated from rats whose testes had been heated to 43°C for 30 min 4 h earlier, there was greater incorporation of labelled methionine into secreted proteins at stages VI–VIII, with no change with tubules at stages II–V and IX–XII. In contrast, with tubules isolated 24 h after heating, incorporation was reduced into tubules at stages VI–VIII, with a lesser effect for tubules at stage IX–XII and no effect for those at stages II–V. Similar changes were seen in intracellular proteins, and reproducible decreases were seen in the secretion of three androgen-regulated proteins (McLaren *et al.*, 1994). The translation of mRNA for histone H1t in pachytene spermatocytes was reduced in tubules incubated at 37°C, 42.5°C or 44°C, whereas only minimal effects were seen on the translation of mRNA for transition proteins 1 and 2 in elongated spermatids; incremental reductions were also seen in the size of the polysomes translating H1t mRNA and sulfated glycoprotein 2 (also known as clusterin) in Sertoli cells, while the size and proportion of polysomal protamine 2 mRNA in elongated spermatids were unaffected (Cataldo *et al.*, 1997). Culturing rat or mouse Sertoli cells for 48 h at 41°C led to an increase in clusterin mRNA after 12 h, i.e. appreciably later than the increases seen in heat-stressed human epithelioid cancer cells (Clark and Griswold, 1997).

Heat has a differential effect on the two haeme oxygenase isozymes present in the rat testis, HO-2, which is normally present in greater amounts is unaffected, whereas HO-1, which is normally present in only low concentrations in Sertoli cells, and could not be detected in Leydig cells, shows intense immunoreactivity in both Sertoli and Leydig cells 6 h after heating to 42°C for 20 min. The mRNA for HO-1 shows an increase in testes within 1 h after heating (Ewing and Maines, 1995). Use of a cDNA probe showed that mRNA for HO-1 could be detected in germ cells as well as in somatic cells in the testis, and while heat stress did not change the pattern of distribution among the cell types, it caused a

distortion of the nuclear pattern and diffusion of the transcript signal in the cells (Maines and Ewing, 1996).

The expression of the mRNA for a recently identified cold-inducible RNA-binding protein in cultured mouse germ cells was reduced when culture temperature was raised from 32°C to 37°C, and when mouse testes were heated, either by making them cryptorchid or immersing in a water bath at 42°C for 30 min, expression was reduced within 6 h of either treatment (Nishiyama *et al.*, 1998).

DNA synthesis in testicular germ cells in isolated seminiferous tubules is much more sensitive to heat than are protein or RNA synthesis (Nishimune and Komatsu, 1972), and later studies have shown that the activity of DNA polymerase beta decreased by 43% within 5 days of the testis being made cryptorchid and the activity of DNA polymerase gamma was even more sensitive (46% decrease after 3 days), whereas the activities of DNA polymerase alpha and topoisomerase I were unaffected (Fujisawa *et al.*, 1988). When minced testis tissue was incubated at 31°C or 37°C, the activities of all four enzymes were lower at 37°C than at 31°C (Fujisawa *et al.*, 1997). Incorporation of thymidine into DNA by human testis fragments was maximal at 31°C, whereas uridine incorporation into RNA and leucine incorporation into protein were maximal at 37°C (Nakamura *et al.*, 1988). One of the most marked changes seen after cryptorchidism involved an extractable enzyme system from mouse spermatocytes capable of catalysing recombination *in vitro*; the activity of this system increases 400-fold between preleptotene and mid-pachytene stages under normal conditions, and is decreased 50-fold after the testes had been cryptorchid for 24 h, and fell even further (350-fold) by the fifth day (Hotta *et al.*, 1988). Unfortunately, shorter times were not examined, but it would be interesting to see how quickly a change in this system could be noticed.

A number of other enzymes in the testis appear to be affected by heat, but again it is not always clear whether this is a direct effect, or a result of the cellular changes within the tissue. For example, uridine kinase, which is normally present in highest amounts in peritubular cells and primary spermatocytes, with less in Sertoli cells, spermatids and spermatozoa in that order, is unchanged after 1 day, but increased after 3 days, and peaks at 12 days after the induction of cryptorchidism in adult rats. Cryptorchidism induced at 17 days of age prevents the normal increase seen as the animal matures (Haugen *et al.*, 1988). Similarly, adenylate cyclase and protein carboxyl methylase do not increase normally if the testis is made cryptorchid at 17 days, but do increase to normal values after orchidopexy when the rats were 34 days old (Jahnsen *et al.*, 1986). The enzyme cholesteryl ester hydrolase which is present mainly in Sertoli cells in rats is reduced in testes made cryptorchid 24 h earlier (Hoffmann *et al.*, 1989).

Insulin-like growth factor I (IGF-I) receptors, which are normally present on primary spermatocytes, Sertoli cells and Leydig cells usually decrease as the rats mature, but if the animals are made cryptorchid at 3 days old, IGF-I receptors increase as the animal ages, mainly due to very large numbers under these conditions in Sertoli cells (Antich *et al.*, 1995).

Temperature may also affect the permeability of the

plasma membranes and lysosomes of germinal cells in the testis, as shown by increased leakage of their cellular contents and increased staining with trypan blue during incubation *in vitro* (Lee and Fritz, 1972; Lee, 1974).

At least some of these changes may result from impaired detoxification of reactive oxygen at the higher temperatures. A reduction in superoxide dismutase and catalase activities were seen 1 day after the testis was made cryptorchid, although the level of oxidative stress, as measured by the appearance of conjugated diene double bonds in polyunsaturated fatty acids was normal after 1 and 3 days, and only increased on day 7, at which time GSH peroxidase, GSH transferase and hexose monophosphate shunt activities were also increased. Superoxide dismutase activity was reduced by incubating normal decapsulated testes at 37°C or 40°C, whereas the enzyme from liver and other enzymes from the testis were unaffected (Ahotupa and Huhtaniemi, 1992). Later studies showed that the abdominal testis before normal descent also demonstrates a higher degree of lipid peroxidation, and the normal decrease is prevented if the testis is made cryptorchid; under these conditions, the oxidative stress in the cryptorchid testis cannot be explained by inactivation of antioxidant enzymes (Peltola *et al.*, 1995).

There has been a great deal of interest recently in heat-shock proteins, which are synthesized by many cell types in response to stress, particularly heat stress. However, in the testis, which contains a high content of these proteins, the major proteins appear to be concerned with development of germ cells, and are not heat inducible. Spermatocytes contain hsp 70-2 and spermatids contain hsp 70t, and neither of these is heat-inducible (see Dix, 1997 for review). However, there is some evidence that some other heat shock proteins (HSI 36 and HSID 74) are formed in greater amounts at higher temperatures (Lemaire and Henlein, 1991). The expression of hsp 70 related gene in the rat testis is not altered in testes that have been cryptorchid for 1 day, although there are decreases at 2, 3 and 6 days (Krawczyk *et al.*, 1987). The expression of hsp 70 was not increased in rats subjected to whole body hyperthermia (42.5°C) for 45 min, although there was a marked increase in livers from these animals (Krawczyk *et al.*, 1988). However, this may be because the principal hsp 70 in the testis is hsp 70-2, which is not heat inducible, whereas hsp 72 is induced in somatic cells in the testis by heat (Zakeri *et al.*, 1990). There is also evidence for an increased synthesis of hsp 70, but not hsp 70-2 (named P70 by these authors) in mouse pachytene spermatocytes when they are subjected to a temperature of 42.5°C for 10 min *in vitro* (Allen *et al.*, 1988a,b). Others have found evidence for induction of two other heat shock proteins, HSP 90 and HSP 27 in the testis in response to heat (decapulated testes exposed to 42°C for 1 h *in vitro*), and also relocation of these proteins to the nucleus. There was also a sharp increase in the labelling of the HSPs in the chromatoid body of round spermatids after hyperthermic treatment (Biggiogera *et al.*, 1996). It has also been shown that mixed germ cell preparations and isolated pachytene spermatocytes from mouse testes exhibited a lower activation temperature for Heat Shock Factor 1 (HSF 1) than did liver cells, and this activation leads to increased expression of hsp 72 in germ cells (Sarge, 1995; Sarge *et al.*, 1995). Another heat shock

factor, HSF-2, is also present in mouse testis, but this appears to be involved in the regulation of the non-inducible hsp 70.2 gene (Sarge *et al.*, 1994).

Another protein that appears to be involved in meiosis is p53, and this gene is associated with the nuclear envelope, and particularly of pachytene spermatocytes (Almon *et al.*, 1993; Schwartz *et al.*, 1993; Yin *et al.*, 1997). Mice deficient in this gene exhibit a giant-cell degenerative syndrome in the testis (Rotter *et al.*, 1993) and temperature-mediated germ cell loss in the testis is associated with altered expression of p53 (Socher *et al.*, 1997). However, p53-deficient mice still showed a fall in testis weight after cryptorchidism, although the decrease was delayed from 7 days to 10 days (Yin *et al.*, 1998).

Endocrine function. The effects of whole body heating on the testosterone concentrations in blood depend largely on how stressful the environment has been to the animal. Young male rats exposed for 3 to 5 weeks to a temperature of 33–35°C have lower body weights and serum testosterone concentrations, although testis weight is not affected (Bedrak *et al.*, 1980). Similarly, bulls exposed to a hot environment for 7 weeks (Rhynes and Ewing, 1973) or 15 days (Minton *et al.*, 1981) showed reductions in serum testosterone concentrations, although in the longer experiment, concentrations returned to normal with time and the response to GnRH infusion was normal. In rams, testosterone concentrations in testes and spermatic venous blood were reduced by 14 days exposure to an environment which varied between 28°C and 32°C each day (Gomes *et al.*, 1971). In boars subjected to an environment at 35°C for 24 h, there was no change in testosterone concentrations in blood, although the diurnal rhythm was lost (Stone and Seamark, 1984). Longer exposure times (100 h at 35°C) caused a fall in testosterone concentrations, with an increase after the period in the climate room; blood cortisol concentrations were significantly increased only during the heat exposure (Larsson *et al.*, 1983). Even longer exposures (34.5°C for 8 h and 31°C for the rest of the day for 14 days) caused a halving of blood testosterone concentrations (Wettemann and Desjardins, 1979).

Cryptorchidism caused serum testosterone concentrations to fall slightly or remain the same in rats, but LH concentrations rose, suggesting that the Leydig cells needed to be driven harder to achieve the same result (Keel and Abney, 1980; Amatyakul *et al.*, 1971; Gupta *et al.*, 1975; Risbridger *et al.*, 1981; Jansz and Pomerantz, 1986). Earlier observations using the weight and composition of the androgen-responsive accessory glands as an index of androgen concentrations suggested that there was no fall in androgen secretion after cryptorchidism, although the evidence for a slight increase at later times seems rather equivocal (Clegg, 1960). However, in rats made unilaterally cryptorchid at birth, androgen concentrations in the cryptorchid testes were much lower than in scrotal testes (Bergh *et al.*, 1984). In cryptorchid rams, testosterone concentrations in peripheral and spermatic vein blood were normal, but LH concentrations were increased, and there was a diminished response to injected LH (Hochereau-de Reviers *et al.*, 1979; Barenton *et al.*, 1982; Lunstra and

Schanbacher, 1988). Testosterone concentrations of short-scrotum rams were generally similar to those of intact animals (Tierney and Hallford, 1985), except when the testes were growing most rapidly during short-day periods (Sanford *et al.*, 1993).

Scrotal insulation in bulls had little effect on blood testosterone or LH concentrations (Prabhakar *et al.*, 1990; Sidibe *et al.*, 1992). In rams, scrotal insulation for 28 days caused severe testicular degeneration, but after 7 days, mean testosterone concentrations in blood were unaffected, while LH concentrations were raised and there was an increase in the number and amplitude of LH pulses. After 14 days mean and basal concentrations of testosterone were lower and the testosterone peaks reduced in size, while LH peaks and mean LH were increased (Byers and Glover, 1984). The effect of scrotal insulation in boars on testosterone concentrations depended on the age of the animals used in the experiments; with mature animals, there was no change in testosterone concentrations for the first 24 h of a 100 h insulation, but thereafter, concentrations fell to almost half of control values. Oestradiol concentrations were also slightly reduced at the same time, but while testosterone concentrations returned to normal after 3 days, oestradiol concentrations remained low for at least 6 days. Oestrone sulphate concentrations responded in a similar way to those of testosterone. In immature boars, neither testosterone nor oestradiol, nor oestrone sulphate concentrations was affected by scrotal insulation for 100 h (Malmgren, 1990).

Local heating of the testes of rats for 30 min to 43°C had no effect on testosterone concentrations in peripheral blood between 2 and 56 days after heating, although the response to LH or hCG was reduced (Damber *et al.*, 1980; Galil and Setchell, 1988a). Between 7 and 35 days after heating, the concentration of testosterone in testicular venous blood was actually higher than control values, although because at that time blood flow was decreased, testosterone secretion was normal or only slightly reduced (Galil and Setchell, 1988b). Raising the temperature of the testis to 41°C or 43°C at the time of an LH injection caused a reduced response to LH in plasma and testis testosterone concentrations, while at a temperature of 37°C, the response was not different from those of controls held at 33°C (Damber and Janson, 1978).

Spermatozoa in rete testis fluid

Two studies have examined the spermatozoa in rete testis fluid collected from testes that had been heated, one study was in rats (Setchell and Waites, 1972) and the other in rams (Setchell *et al.*, 1971; Voglmayr *et al.*, 1971). In rats, the concentration of spermatozoa in rete testis fluid began to decrease within 10 days after a single exposure of the testes to 41°C for 60 or 90 min, and returned to normal at about 38 days; the fall was about 1000-fold. In rams, the decrease occurred later, about 20 days after heating to 40°C for 3 h, although the concentrations returned to normal at about the same time as in rats. There was also a decrease in the rate of flow of rete testis fluid in rams during the heating period, and there were changes in the metabolism and ultrastructure of the spermatozoa collected within 1–4 days after heating.

The fall in sperm concentration in rams was greater than in rats. The timing of the fall was consistent with a major effect on pachytene spermatocytes and early spermatids.

Spermatozoa in semen

Increase in body temperature in men by whole body heating (MacLeod and Hotchkiss, 1941), or local heating of the testes (Watanabe, 1959; Tokuyama, 1963) is followed by a decrease in sperm concentration in semen between 3 and 11 weeks later. After sauna baths (Procope, 1965; Brown-Woodman *et al.*, 1984), after local heating of the testes (Robinson *et al.*, 1968) or after self-induced scrotal hyperthermia (French *et al.*, 1973), there was also a fall in the number of spermatozoa, in some instances within 1 week after heating, but never earlier than that.

In bulls, whole body heating produced decreases in numbers and motility of spermatozoa, and a rise in abnormal and dead cells, also beginning 1 week or more after a single exposure, and persisting for between 40 and 60 days after the end of the heating (Casady *et al.*, 1953; Skinner and Louw, 1966). In rams, decreases in motility and in the percentage of normal spermatozoa and, in some cases, numbers of spermatozoa occurred between 1 and 2 weeks after heat exposure, returning to normal after between 30 and 42 days (Dutt and Hamm, 1957; Moule and Waites, 1963; Smith, 1971). In boars, in a number of experiments, the number of spermatozoa was not affected, but motility and the percentage of normal spermatozoa fell between 1 and 6 weeks after the heat exposure; when the heating was for only a limited period, motility and the percentage of normal spermatozoa recovered after about 6 weeks (McNitt and First, 1970; Wettemann *et al.*, 1976, 1979; Stone, 1981; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984).

Local heating of bull testes by insulation also caused falls in motility and percentage of normal spermatozoa, beginning 12 to 14 days later, and lasting until day 26 to 90, depending on the length of insulation. There were also decreases in sperm concentration in most experiments, beginning usually 1 to 2 weeks later than the changes in motility (Austin *et al.*, 1961; Ross and Entwistle, 1979; Wildeus and Entwistle, 1986; Vogler *et al.*, 1991, 1993; Karabinus *et al.*, 1997). Even insulation of only of the neck of the scrotum produced similar effects (Kastelic *et al.*, 1996). In rams, local heating of the testes or insulation of the scrotum caused similar changes in sperm motility and the percentage of normal spermatozoa, and if maintained continuously for a sufficient period, in the concentration of spermatozoa in semen; intermittent insulation often caused decreases in motility without any changes in the number of spermatozoa (Glover, 1955; Waites and Setchell, 1964; Braden and Mattner, 1970; Mieusset *et al.*, 1992a). In pigs, insulation of the scrotum for 100 h had no effect on the number of spermatozoa, but motility and the percentage of normal spermatozoa decreased between 2 and 6 weeks afterwards (Malmgren, 1989). In stallions, insulation for 24 or 48 h produced falls in motility after only a few days, with the number of spermatozoa and the percentage of normal spermatozoa falling after about 10 days; recovery occurred at about 70

days (Freidman *et al.*, 1991). In rats in which the accessory glands had been removed surgically so that the ejaculate could be liquefied with chymotrypsin, the number of spermatozoa in semen obtained by electroejaculation fell about 10 days after immersion of the testes in a water bath at 41°C for 27 min and remained low until about day 70 (Maus, 1971). In mice whose testes had been heated to 40°C for 60 min, the spermatozoa in the cauda epididymidis showed an increased percentage of abnormal forms from day 7 to day 35, and the structure of the sperm chromatin was abnormal, as judged by the susceptibility of the epididymal spermatozoa to *in situ* denaturation at low pH, from day 3 to day 35; mice exposed to 42°C for 60 min had too few spermatozoa in the epididymis for measurements to be made (Sailer *et al.*, 1997). Mice whose testes were heated to 42°C for 20 min still had spermatozoa in the epididymis 28 days later, but there were fewer than in controls, and motility was decreased (Jannes *et al.*, 1998).

Fertility and fertilization

The effects of exposure to heat on the fertility of male animals has been known for a long time, although the effects vary widely between individual animals, and depend on the extent of acclimatization. Young (1927) found that of 19 male guinea-pigs whose testes were heated to the same extent, two continued to reproduce normally, but the other 17 showed various effects, ranging from temporary sterility lasting from 7 to 44 days after heating to permanent sterility in three individuals. Cunningham and Osborn (1929) heated the testes of rats with infra-red or hot air, and found that the animals were sterile between 16 and 82 days and 34 and 85 days, respectively, with a second sterile period following a short period of normal fertility lasting about 20 days. Elfving (1950) heated the testes of rats in a water bath at 44.3°C for 20 min, and found that all animals became sterile. In three, sterility was permanent, in the other 12, the sterile period began between 10 and 20 days after heating and ended between 50 and 140 days after heating. Furthermore, he found with a small number of animals that the onset of the sterile period could be delayed by treatment with serum gonadotrophin (a crude preparation of FSH), thyroid hormone or a low dose of testosterone; hCG or a large dose of testosterone was without effect. None of the treatments tried altered the time of restoration of normal fertility. In a later study with rats, the infertile period after heating the testes to 43°C for 30 min lasted from between 7 and 14 days after heating to between 49 and 56 days (Setchell *et al.*, 1988). When the testes of mice were heated to 42°C for 20 min, they remained fertile, but if the heating period was extended to 30 min, although they continued to mate at a normal rate, no pregnancies resulted between 10 days and 32 days with one male and 38 days with the other (Setchell *et al.*, 1998). When mice were placed as adults in an environment at 32.7°C or 36.1°C for 2 weeks, 100% became sterile at the higher temperature, but only 43% under the less severe conditions. However, if the mice had been exposed to the hot conditions from weaning, 83% and 67%, respectively were fertile at the lower and higher temperature when they reached adulthood

(Pennycuik, 1967). In another study, transferring adult mice from 21°C to 35°C and 65% humidity for 24 h had no effect on their fertility, but exposure for 48 h reduced the percentage of pregnant females from 66% to 36%, falling to 27% after 96 h and 18% after 120 h (Garriott and Chisman, 1981).

Burfening *et al.* (1970) exposed male mice to a chamber maintained at 32°C with 65% humidity for 24 h, and then mated them with normal females. Embryos were recovered 48 h after a copulation plug had been found, and fertilization rate calculated from the ratio of cleaved ova to total ova recovered. They found that fertilization rate fell from 79% between days 1 and 5 after heating to 61, 43 and 11% for days 6–10, 11–15 and 16–20, respectively, and then recovered to 55% and 79% for days 21–25 and 26–30 after heat treatment; control males had fertilization rates of between 78 and 91% over the same periods. In rats whose testes had been heated to 43°C for 30 min, the fertilization rate of eggs collected the morning after mating was reduced from 76% to 17% 16 to 20 days later, but there were fewer spermatozoa in the uterus of the non-pregnant females (Setchell *et al.*, 1988). Dutt and Simpson (1957) noted that the fertilization rate in ewes increased by 38% when rams were kept in air-conditioned chambers during the summer months. Heating the testes of rams to 40.5°C for 2 h or 39.5°C for 4 h led to low fecundity between days 14 and 34, and zero between days 34 and 47 (Braden and Mattner, 1970). Similarly, exposure of rams to an ambient temperature of 32°C and relative humidity of 65% for 4 days was followed by severely reduced fertility in week 2 and no pregnancies in week 3 (Howarth, 1969). When rams were exposed to 40.5°C for 8 h, on one, two, three or four occasions on successive days, and then each mated between 10 and 27 days later to eight superovulated ewes, 93% of the eggs recovered 60–70 h after mating were fertilized by the control ram, whereas the percentage fell to 66, 42, 23 and 6% respectively for the increasing times of heat exposure (Rathore, 1970). When boars were exposed to 34.5°C for 8 h and 31°C for 16 h per day for 6 weeks, and over the next 5 weeks were either allowed to mate with normal sows, or semen was collected and 6×10^9 spermatozoa per sow used for artificial insemination, 59% of the sows became pregnant by natural mating and 29% by artificial insemination, compared with 82 and 41%, respectively for control boars (Wettemann *et al.*, 1976, 1979). When ewes were inseminated in each uterine horn with 50×10^6 frozen-thawed spermatozoa from 16 h per day scrotal-insulated rams, the pregnancy rates at 17 days were not different from control values (Mieusset *et al.*, 1992a).

However, with the exception of the experiments involving artificial insemination, most of these results could be explained by a reduced number of spermatozoa being available to fertilize the eggs, and even in the AI experiment, no allowance was made for the fact that the semen samples from the heated animals had lower motility than those of the controls. Nevertheless, when an equal number of motile swum-up spermatozoa from scrotal-insulated and control rams were used for *in vitro* fertilization, using ova obtained from ovaries collected at an abattoir, the percentage of eggs fertilized decreased from 73% to 7% with 16 h per day insulation for 18 days, and there were significant falls with

8 h per day insulation for 5 weeks, both during the insulation period and during the following 2 weeks (Ekpe *et al.*, 1992, 1993; see also Setchell 1994). AI was also undertaken with epididymal spermatozoa from mice whose testes had been heated to 42°C for 20 min 28 days earlier. Fertilization rates were reduced (45% of oocytes fertilized compared with 65% for controls), but although the number and motility of spermatozoa were reduced (Jannes *et al.*, 1998), the authors do not make it clear how many motile or total spermatozoa were used in the IVF.

Embryonic development

Probably the most surprising aspect of the effects of heat on the male is the possibility that there may be an effect on the development of embryos, even when the ova come from a normal female. In the experiments on guinea-pigs reported by Young (1927), he noted that there was an increase in the percentage of stillborn and aborted pups sired by the heated males when the proportion of sterile mating rose. For example, in the first 7 days after heating, only 7% of the matings were sterile and 7% of the pups were stillborn or aborted. In the second period, which extended from 8 days after heating to 48 days, 28% of the matings were sterile, and 25% of the pups were aborted or stillborn. Surprisingly, Young did not consider this rise in the proportion of abnormal pups to be necessarily a direct effect of the heating. Dutt and Simpson (1957) noted that embryonic death rate fell by 27% when rams were kept in air-conditioned chambers during the summer months. In the experiment by Burfening *et al.* (1970), for which the fertilization rates are given above, the authors also autopsied other females at day 10.5 of pregnancy and noted the number of corpora lutea, implantation sites and normal fetuses. From their data, it is possible to calculate the ratio of implantation sites to fertilized ova, and of fetuses to implantation sites. The latter remained between 86 and 96% for the heated males and 92 and 98% for the controls, but the ratio of implantation sites to fertilized ova was already less for the heated males in the first 5 days (71% versus 90%), and continued to fall to 58% for days 6–10 and to 55% for days 11–15, while the values for control males stayed at 89% and 88% for the same periods. This suggests that even epididymal spermatozoa may be affected as well as cells developing in the testis in such a way as to affect the developmental capacity of the embryos they produce, as the matings achieved on days 1–5 would involve spermatozoa already in the epididymis. This was also the case in the experiments reported by Mieusset *et al.* (1992a); ewes inseminated with frozen-thawed semen collected from rams 4 days after the beginning of scrotal insulation showed a greater loss of embryos between day 17 and day 65 of pregnancy, although the loss became more pronounced when the semen was collected 11 or 18 days after the start of insulation.

Bellve (1972, 1973) found that following exposure of male mice to an ambient temperature of 34.5°C and 65% humidity for 24 h, that there was an extensive embryonic mortality evident by day 10.5 of gestation and developmental retardation, as evidenced by an accumulation of four-cell

embryos at 54 h after coitus when the males were mated to normal females 6 days later. The eight-cell embryos sired by heated males were less able to incorporate [^3H]uridine. Bellve also found reduced numbers of blastocysts *in vivo* 120 h after hCG injection and two-cell embryos recovered from normal females and cultured *in vitro* showed a minor arrest at the eight-cell stage, and a substantial accumulation of morulae, so that the number of blastocysts was reduced. When normal female rats were mated to males whose testes had been heated to 43°C for 30 min, before the males became sterile, and during recovery of fertility, there were periods when litter size and the fetus to corpus luteum ratios were reduced, suggesting that there was significant mortality of the embryos. There was also an increased incidence of degenerating embryos at 2 and 15 days of pregnancy (Setchell *et al.*, 1988). However, with male rats acclimatized for 3 months to 35°C, although the mating rate was only 17% and conception rate 40%, compared with 52% and 71%, respectively for controls, the development of the embryos once conceived appeared to be normal (Sod-Moriah *et al.*, 1974).

In the experiments with pigs by Wettemann *et al.* (1976, 1979) for which the fertilization data are given above, an estimate was also made of the survival of the embryos to 30 days after coitus. In the artificially inseminated sows, embryo survival was only 49% with semen from the heated males, compared with 71% with semen from control males. An equivalent difference was not seen in sows mated naturally (79% versus 82%), and the authors suggest that this is because of the larger numbers of spermatozoa inseminated during a natural mating.

There is also some evidence that even the heating of ejaculated spermatozoa can cause increased embryo mortality if they are subsequently used for insemination (Young, 1929; Howarth *et al.*, 1965; Ulberg and Burfening, 1967).

The sheep embryos produced by *in vitro* fertilization, using semen from scrotal-insulated rams developed slightly less well than those obtained with control semen, and there was increased degeneration at the blastocyst stage (Ekpe *et al.*, 1992, 1993; see also Setchell 1994).

When the testes of male mice were heated to 42°C for 20 min, although the animals remained fertile (see above) and litter size was not reduced, the embryos they sired were about 25% smaller than controls at 10.5 days of gestation in three separate experiments with different strains of mice; at later stages of pregnancy, the difference became smaller, but in one experiment, although not in a second, the differences were still significant at 15.5 and 18.5 days. The difference did not appear to be due to later mating by the heated males, and the timing of the growth retardation differed from that seen in mice lacking IGF-II, of which only the paternal copy is expressed in the embryo (see Setchell *et al.*, 1998). These findings of smaller fetuses with normal litter size from heat-exposed sires have been confirmed by Jannes *et al.* (1998), for embryos at 14.5 days. This finding was not entirely unexpected, in view of the findings that human semen of poor quality, not necessarily caused by heat, appears to produce poor quality embryos (Ron-El *et al.*, 1991; Parinaud *et al.*, 1993; Janny and Menezo, 1994).

Mechanism of effects of heat

Because of the wide range of effects of heat on the testis, as described above, it is difficult to propose a single cause. Either a variety of cell types are affected in different ways, or one cell type is primarily affected, and there are secondary effects on the other cells. As the Leydig cells do not appear to be directly affected by heating, or if they are, are only minimally affected, the most obvious cell to be the primary site of action of heat is the Sertoli cell. Because of its position in the seminiferous epithelium, it is able to have a profound influence on all germ cells once they pass through the blood–testis barrier. These germ cells probably depend almost entirely on the Sertoli cells for nutrients and their development is controlled by influences from the Sertoli cells. The Sertoli cells probably even have an influence on cells such as the spermatogonia and preleptotene spermatocytes that are in the basal compartment, on the blood side of the specialized junctions between the Sertoli cells, which form the major site for the barrier inside the tubules.

However, there is very little direct evidence that the Sertoli cells are affected by heat. Secretion of fluid does appear to be reduced under some circumstances, but not in the first 24 h after the testis has been made cryptorchid. However, there may be more subtle changes in composition of the secreted fluid not reflected in total volume secreted and it would seem logical to examine the composition of the secretion by these cells at different temperatures, as the secretion contains high concentrations of many substances that are either not found elsewhere in the body, or are present at much lower concentrations. An effect on the Sertoli cells could influence chromosome behaviour during the meiotic prophase, and investigations on the effects of heat on the synaptonemal complex may be warranted.

The only effects that cannot be explained by a primary effect on the Sertoli cells are those involving the spermatozoa in the epididymis. Although these are not major, there does appear to be evidence for them, although most authors agree that epididymal spermatozoa are much less susceptible. However, it has been suggested that the epididymis is the prime mover in the evolution of the scrotum (Bedford, 1978), and these effects of heat on epididymal spermatozoa merit further investigation. In addition, as when the spermatozoa leave the testis they are incapable of fertilizing ova, unless injected directly into the egg cytoplasm, an epididymal effect could contribute to the reduction in capacity of the spermatozoa, although motile, to fertilize.

The fact that blood flow through the testis does not increase at all, or not sufficiently to match the increase in metabolism, means that the heated testis is probably hypoxic. Damage may be caused not so much by the hypoxia directly, as by the generation of reactive oxygen species during the recovery phase, as occurs after ischaemia in many other tissues and the effect of scavengers for ROS during heating or immediately afterwards is probably worth investigating.

The many changes in enzymes and in heat shock factors and heat shock proteins must be evaluated to determine whether these responses are deleterious or are an attempt by

the testis to protect itself against the effects of heat. The latter is probably the case with the haeme oxygenase enzymes.

As there is now evidence that the contribution of the paternal and maternal gamete to the embryo is not the same, so-called 'genomic imprinting' (see Ohlsson *et al.*, 1995), it seemed possible that heat may have been interfering with the process whereby the paternal genome is identified as such. This does not appear to be the case for the imprinted gene most obviously concerned with fetal growth, IGF-II, but there are now many such genes, and it is possible that the imprinting signal is not being properly added to the spermatozoa. There is also evidence for non-genomic contributions by the spermatozoa to the embryo (Browning and Strome, 1996), and it is possible that the factor or factors may be influenced by temperature.

Conclusions

Although it is not yet possible to explain why spermatogenesis is so sensitive to heat, the fact remains that heat or summer sterility is recognized as a major problem in farm animals (see Setchell, 1978). More recently, there has been evidence provided that in humans, increased testis temperatures, either due to occupational hazards or clothing fashions may be contributing significantly to infertility or subfertility problems (Mieusset and Bujan, 1995; Parazzini *et al.*, 1995; Thonneau *et al.*, 1997). Heating has also been considered as a possible contraceptive technique (see Kandeel and Swerdloff, 1988). It has even been suggested that global warming may be a possible cause of the decline in human sperm counts reported by some authors. If this is the case, it is surprising that similar falls have not been detected in any of the farm animals, for which much more data have been collected, and which would have been much more directly influenced by climatic change (see Setchell, 1997; van Os *et al.*, 1997).

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