

Graafian follicles are cooler than neighbouring ovarian tissues and deep rectal temperatures

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In order to establish appropriate culture temperatures for in-vitro maturation of pig ovarian oocytes, large Graafian follicles (7–10 mm diameter) were sensed by infra-red technology during the latter part of a spontaneous oestrous cycle. Temperatures were measured under systemic anaesthesia almost instantaneously upon revealing the ovaries at mid-ventral laparotomy. Temperature differentials were observed within all 16 ovaries sensed in 14 animals. Ovaries were always cooler than deep rectal temperatures (mean rectal temperature was $38.0 \pm 0.4^\circ\text{C}$; range $37.5\text{--}38.6^\circ\text{C}$) and mature follicles always cooler than ovarian stroma ($35.6 \pm 0.3^\circ\text{C}$ versus $37.3 \pm 0.2^\circ\text{C}$ respectively; $P < 0.01$). Such follicles were frequently $1.5\text{--}1.8^\circ\text{C}$ cooler than the adjacent stroma, the mean being $1.7 \pm 0.4^\circ\text{C}$. Small Graafian follicles ($<5\text{--}6$ mm diameter) and recent ovulations did not show this differential. The control experiment of excising an ovary, deep freezing it in liquid nitrogen, and then restoring it to the body cavity before further sensing indicated that intra-ovarian temperature gradients depended on the activity of living tissues and/or a functional blood supply. Furthermore, calculation of anticipated rates of cooling for exposed Graafian follicles strongly suggested that artefacts could not have been solely responsible for the observed temperatures. Endothermic reactions within mature follicles were thus brought into focus. It is concluded that follicular temperatures may influence the meiotic progression and cytoplasmic maturation of oocytes and act to regulate enzymatic activity in the biosynthetic pathways for steroid and/or peptide hormones.

Key words: Graafian follicles/oocytes/ovary/pig/temperature gradient

Introduction

For fairly obvious reasons, there is widespread appreciation of the fact that male gonads need to assume a cooler, scrotal location by early post-natal life if they are to become fully functional at puberty and produce competent spermatozoa. As judged from experiments that involve surgical restoration of testes to the abdomen (Griffiths, 1893; Glover, 1960), or from nature's own experiment of cryptorchidism (John Hunter,

1786), prolonged exposure to full abdominal temperature is damaging to the germ cell epithelium and can soon render an animal infertile and eventually sterile. Nonetheless, a small but significant proportion of the relatively few eutherian mammals so far examined have abdominal testes, notable examples being the elephant, hyrax, and various marine species (Millar and Glover, 1973; Harrison, 1975; Glover, D'Occhio and Millar, 1990). The fact that such testicond mammals demonstrate normal spermatogenesis and are fertile raises the interesting question as to whether such testes function at full abdominal temperature and if there are local physiological mechanisms that impose some degree of cooling, even deep within the abdominal cavity. These problems need to be addressed by means of modern technology.

Broadening the discussion to one of overall germinal strategies, there is the related question of whether female gonads consistently function at full abdominal temperature or if compartments within an ovary, especially those concerned with maturing gametes, achieve some form of temperature gradient at appropriate stages of the oestrous or menstrual cycle. In other words, and despite the general notion of a mid-cycle increase in deep body temperature in primates, it is worth considering seriously whether this is specifically so for the Graafian follicle. Such a mature follicle is largely responsible for the underlying endocrine changes that prompt responses in target tissues and yet it will soon liberate a potentially fertile oocyte. In fact, previous reports from Copenhagen indicated that rabbit pre-ovulatory follicles were $1.4 \pm 0.2^\circ\text{C}$ cooler than ovarian stroma when examined by means of infra-red scanning or microelectrodes inserted during mid-ventral laparotomy (Grinsted *et al.*, 1980). The antral fluid temperature of human follicles was also cooler than ovarian stroma, on occasions by as much as 2.3°C (Grinsted *et al.*, 1985). Also, by the relatively crude technique of thermistor probing, studies in Edinburgh on pig Graafian follicles of 8–10 mm diameter suggested that the antrum was at least 1°C cooler than the neighbouring stroma or Fallopian tube tissues (unpublished observations). In the present study, we have used the latest infra-red technology to measure temperature differentials within the ovaries of fully mature animals with spontaneous oestrous cycles. The findings raise a number of exciting physiological interpretations and perplexing questions. The temperature gradients have been reported briefly to the Society for the Study of Fertility (Hunter *et al.*, 1995).

Materials and methods

Experimental animals

In all, 16 Large White gilts weighing ~85–98 kg and aged 5.5–7 months were purchased from a local breeding farm. They were

housed as groups of 3–5 animals in open-fronted indoor pens in the Department of Veterinary Clinical Studies, bedded on straw, and fed a standard pelleted diet twice daily; water was available *ad libitum*. Natural daylight was supplemented with artificial illumination at feeding time and during clinical examination. A mature Duroc boar was held in an adjoining pen. Hormone preparations were not administered.

Gilts were checked three or four times daily for the stage of their oestrous cycles, especially that of late pro-oestrus. Particular attention was paid to vulval swelling, colour, and the nature of escaping secretions. Behavioural interactions in front of the boar were also closely monitored.

Pre-operative procedures

At selected stages during the follicular phase of a 20 or 21 day cycle, or exceptionally during pro-oestrus of the first recorded cycle, animals were isolated overnight in starving pens with an insulated floor. On the following morning, they were walked across the isolation corridor into the heated operating suite, a distance of <30 m. Premedication had not been given. They were restrained with a snout rope, injected i.v. with propofol (Diprivan; 10 mg/ml; Zeneca Ltd) to induce anaesthesia, lowered onto a stretcher, and lifted onto a padded and heated operating table (26°C). Endotracheal intubation was achieved with the animal on its side using a Rowson laryngoscope (Penlon Ltd, Andover, Hants, UK) and cuffed McGill tube, after which anaesthesia was maintained with semi-closed circuit administration of isofluran (Forene; Abbott Labs. Ltd.) and oxygen. As soon as full relaxation was achieved, the animal was positioned on its back, a deep-rectal temperature probe inserted (newly-calibrated for each animal against four medical thermometers), and the operating field on the abdomen cleaned, sterilized and fully-draped. Ambient temperatures in the theatre were also recorded.

Operative technique and temperature measurement

Strict aseptic procedures were followed throughout. A rapid mid-ventral laparotomy was performed, the incision being 16–18 cm in length. The fascia was parted by blunt dissection and the body wall was opened in the mid-line. Before displacing the sub-peritoneal fat and entering the abdominal cavity, the infra-red camera was positioned and focused immediately above the incision, supported on an independent, fully-swivelling tripod.

The infra-red camera (model Thermovision 470, Agema, Copenhagen, Denmark) and associated computing equipment were made available by Praecisions Teknik (Copenhagen, Denmark). Calibration had been achieved to an accuracy of $\pm 0.1^\circ\text{C}$. During actual recording, temperature gradients could be visualized in colour on a video monitor screen adjoining the operating table. Such information could subsequently be generated on a computer screen, enabling precise pin-pointing and measurement of individual follicular temperatures and that of neighbouring stroma. All infra-red measurements and subsequent down-loading of the information were made by the same individual (CG) using a Compact Contura computer model 400C system with Irwin 2.0.

Infra-red temperature recording began immediately ovaries were brought individually from their pelvic location to the incision site by careful traction on the mesovarium. The fimbriated extremity of the Fallopian tube was displaced. Ovaries were visualized by manually reflecting the body wall and peritoneum laterally from the incision rather than by withdrawing individual gonads to the level of the body surface. An almost instantaneous survey of reproductive tissue temperatures was thereby obtained, with the ovary and adjoining tract still located deep within the incision site. This approach also permitted Fallopian tube temperatures to be sensed swiftly in four of the animals.

After infra-red recording was completed, and inspection and photography of the ovaries, the abdominal incision was closed in three layers. The peritoneum was closed with a continuous suture whereas interrupted sutures were placed in the body wall and skin. An injection of 10 ml phenylbutazon (200 mg/ml) was given i.m. as analgesic. All animals recovered without incident in the post-operative penning of the Clinical Department. None of the animals was operated on a second time.

Results

Ambient temperatures in the operating theatre usually ranged from 24–28°C, most commonly 24–26°C, not in the course of an individual experiment but rather during the 3.5 month period over which the studies were performed.

Ovaries bearing suitable follicles, that is three or more pre-ovulatory follicles or recently-collapsed follicles in the same hemisphere of the gland (and thus accessible for simultaneous scanning), were examined in 16 individual instances. The recorded temperatures are presented in detail in Tables I and II and in Figure 1, and examples illustrated in colour in Figure 2. Ovaries were always cooler than deep rectal temperatures: the mean rectal temperature was $38.0 \pm 0.4^\circ\text{C}$ (range 37.5–38.6°C) whereas the mean ovarian stroma temperature was $37.3 \pm 0.2^\circ\text{C}$, with a range of 35.7–38.5°C. Mature Graafian follicles of 7–10 mm diameter were invariably cooler than ovarian stroma, with a mean temperature of $35.6 \pm 0.3^\circ\text{C}$ and range of 34.5–37.2°C (Table II). Such follicles were most frequently 1.5–1.8°C cooler than the adjacent stroma, although the mean differential was $1.7 \pm 0.4^\circ\text{C}$ cooler (Table II).

Temperatures amongst the population of pre-ovulatory follicles were not uniform (see Table I). Small follicles, regarded as those <5–6 mm diameter, did not reveal mean differentials comparable with those mentioned above. These follicles were generally <1.0°C cooler than stroma and frequently <0.7°C, as was the case for recent ovulations.

As a means of establishing that the surface of Graafian follicles was not being ‘sensed’ by the infra-red camera in a manner that differed from other portions of the ovary, thereby leading to artefacts, a unilateral ovariectomy was performed in two animals immediately after the initial temperature recordings. The excised ovary was suspended on a braided silk suture thread (No. 3 Mersilk; Ethicon Ltd, UK) attached to a portion of the mesovarium and plunged directly into a flask of liquid nitrogen (–196°C) to kill the tissues. It was then withdrawn, thawed in warm water, and returned to the abdominal cavity where it was left to equilibrate for 6–8 min and achieve its original morphology. Finally, it was brought up to the incision site and examined once more by infra-red scanning. In both animals in which this control experiment was performed, there was no difference in temperature between the thawed follicular surface and that of the ovarian stroma.

With regard to artefacts induced by invasive abdominal surgery, cooling of exposed follicles was recorded within 5–8 s. The fall in temperature noted during this interval ranged from 0.4–0.6°C and, in one animal, a loss of 1.7°C was recorded after 11 s of follicular cooling. A theoretical calculation has been made of the rate of cooling of a 9 mm diameter Graafian follicle containing 0.4 ml of extractable follicular fluid, and

Table I. Detailed individual observations in 10 of the animals (gilts) whose ovaries were examined by infra-red scanning during the follicular phase of the oestrous cycle. These 10 animals represented those in which the first ovary to be revealed at surgery contained Graafian follicles suitable for measurement

Animal number	Deep rectal temperature (°C)	Behavioural stage of cycle	Condition of reproductive tract	Diameter of pre-ovulatory follicles (mm)	Temperature of ovarian stroma (°C)	No. follicles scanned on first ovary*	Ovarian follicular temperatures		Follicular temperature below that of stroma	
							Mean (°C)	Range	Mean (°C)	Range
691	38.4	In oestrus	Uterus very tonic	9–10	38.4	6	37.0	36.7–37.2	1.4	1.2–1.7
728	37.7	Onset of oestrus	Uterus very tonic	8–9	36.5	4	34.8	34.4–35.6	1.7	0.9–2.1
745	37.5	Early oestrus	Tract exceptionally tonic	8–9	36.2	3	34.5	34.0–35.1	1.7	1.1–2.2
701	37.7	Pro-oestrus	Relatively immature tract	7	36.1	2	34.8	34.1–35.4	1.3	0.7–2.0
670	38.0	Not yet in oestrus	Not tonic	7	36.6	4	34.6	34.3–35.0	2.0	1.6–2.3
667	38.0	In oestrus	Tract very tonic	9–10	37.8	7	35.7	35.2–36.3	2.1	1.5–2.6
666	38.6	In oestrus	Very tonic and contractile	Just ovulating	37.8	1**	35.8		2.0	
833	38.0	Not yet in oestrus	Reasonably tonic	8–9	38.2	2	35.8	35.6–36.0	2.4	2.2–2.6
914	38.0	In oestrus	Very tonic	9–10	38.2	4	37.2	36.9–37.4	0.9	0.8–1.3
961	38.5	Just in oestrus	Tonic	9	38.0	3	36.4	36.2–36.7	1.6	1.3–1.8

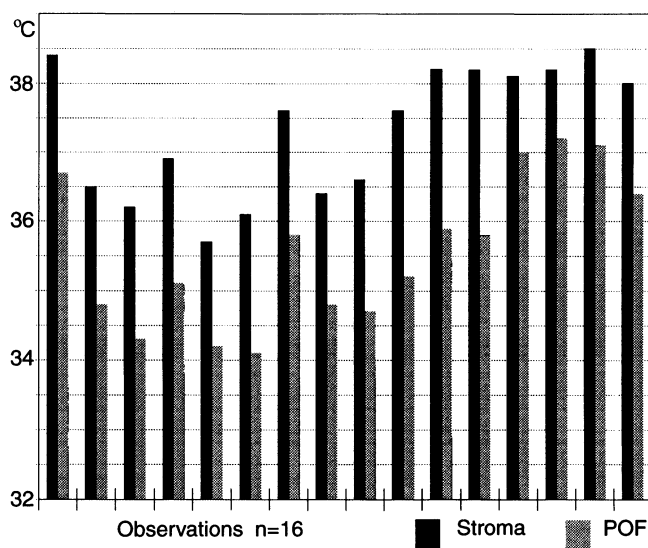
*Ovaries exposed in random sequence. Not all pre-ovulatory follicles examined, simply those that first presented themselves.

**Plus three follicles on this ovary that had very recently collapsed at ovulation.

Table II. Summarized findings concerning deep rectal and ovarian temperatures from 16 sets of observations (ovaries) in 14 animals examined during mid-ventral laparotomy. Almost instantaneous measurements were made by infra-red scanning upon exposure of the ovaries

	Temperature (°C)			
	Deep rectal probe	Ovarian stroma	Graafian follicles 7–10 mm diameter	Follicles cooler than stroma (differential)
Mean ± SE	38.0 ± 0.4	37.3 ^a ± 0.2	35.6 ^b ± 0.3	1.7 ± 0.4
Range	37.5–38.6	35.7–38.5	34.5–37.2	0.7–2.6

^{a,b} Significant difference ($P < 0.01$) when tested by one-way analysis of variance.

**Figure 1.** To highlight the temperature differential between the ovarian stroma and mature Graafian follicles (7–10 mm diameter) during almost instantaneous recording of gonadal temperatures by infra-red sensing at mid-ventral laparotomy in 16 ovaries from 14 animals (POF = pre-ovulatory follicles). Temperatures measured in °C.

50% of whose surface is exposed to a theatre air temperature of 26°C but a theatre wall temperature of 15°C. For the present purpose of illustration, assuming no convection currents within the incision site, no equilibrating influence of blood flow, and no surface moisture to evaporate, such a follicle would be expected to cool at a rate of 1.0°C per 160 s. If a film of moisture (peritoneal fluid) were to evaporate from the surface of the follicle, then the anticipated rate of cooling would become 1.0°C in ~11 s. Within the limits of these calculations, cooling artefacts could not have been solely responsible for the observed temperatures.

Discussion

After measurement of temperature gradients in the Fallopian tubes close to the time of ovulation (Hunter and Nichol, 1986), and with changes in the maturing male and female gametes in mind, it was a logical further step to focus attention on the ovaries in general and on large Graafian follicles in particular. Previous studies in Copenhagen had noted that rabbit pre-ovulatory follicles were 1–2°C cooler than ovarian stroma during infra-red or microelectrode measurements at laparotomy (Grinstead *et al.*, 1980). Human Graafian follicles were as much as 2.3°C cooler than ovarian stroma when examined with

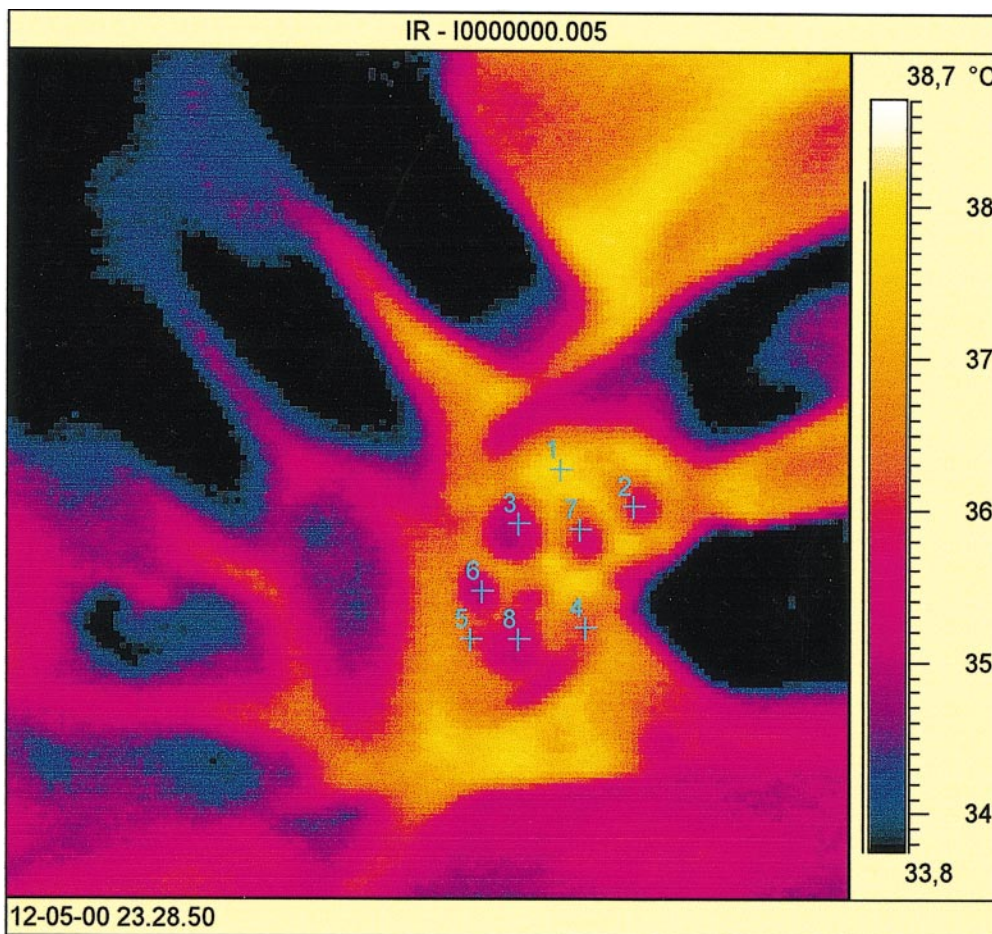


Figure 2. Infra-red sensing of ovarian temperature differentials made during midventral laparotomy with almost instantaneous recording of temperature upon exposure of the ovaries. The coloured scale of temperatures on the right of the picture enables mature Graafian follicle temperatures to be contrasted with those of the adjoining stroma. Graafian follicles of 7–10 mm diameter (blue crosses nos. 2–8) were consistently cooler: 2, 35.9°C ; 3, 35.3°C ; 4, 36.3°C ; 5, 36.2°C ; 6, 35.2°C ; 7, 35.9°C ; 8, 35.3°C (mean difference $1.7 \pm 0.4^\circ\text{C}$) than neighbouring stromal tissues (blue cross no. 1) at 37.8°C The surgeon's fingers appear as black.

thermoelectrodes sited in the antrum (Grinsted *et al.*, 1985) although, as judged from a literature survey, these particular results had seemingly attracted little attention. Studies in Edinburgh using fine thermistor probes in oestrous pigs had also recorded a downhill gradient of $>1^\circ\text{C}$ between the ovarian stroma or neighbouring Fallopian tube and follicles of 9–10 mm diameter, but the findings were never submitted for publication due to concern over possible artefacts. The use of thermistor probes had necessarily involved an invasive technique, requiring puncture of the follicle wall with a consequent modification of the vascular bed. There was the additional disadvantage that the thermistor approach could not provide instantaneous measurements upon surgical exposure of the ovaries. Accordingly, the latest infra-red technology used in the present studies to measure temperature differentials within the ovary was considered a valuable advance.

The current findings on temperature gradients within the mammalian ovary, especially the significantly cooler Graafian follicles during the late follicular phase of the oestrous cycle, require careful evaluation and interpretation. Indeed, at first sight they seem a remarkable observation, although it is worth adding that similar results have now been obtained for bovine pre-ovulatory follicles using the same technology (Grøndahl

et al., 1996). The term 'stroma' has been applied rather loosely. Since, volumetrically, much of the stroma was represented by pale yellow or white corpora albicantia with a negligible vasculature, and such tissue was always warmer than Graafian follicles, this emphasizes all the more strongly the temperature of the follicles. Therefore, the principal question to be addressed must be 'precisely how do ovarian tissues enclosed in the body cavity at, say, 37.5°C or 38°C and perfused by a blood supply presumed also to be at 37.5°C or 38°C , manage to achieve a reduction in temperature?' Several responses come to mind. Firstly, a mature Graafian follicle is predominantly a fluid-filled sac, in the domestic pig of 7–10 mm diameter, with a vascular supply to the thecal layers. Capillaries are able to traverse the basement membrane and vascularize the granulosa cells only after breakdown of this membrane following the pre-ovulatory gonadotrophin surge (Baker, 1972a,b; Baird, 1984; Espey and Lipner, 1994). Thus, in volumetric terms, a mature Graafian follicle is very largely an avascular structure with a relatively static reservoir of fluid when viewed grossly. Secondly, heat might be removed from protruding Graafian follicles by local currents of peritoneal and Fallopian tube fluids generated by the ciliary and muscular activity of the fimbriated extremity of the tube; such currents would have

much less impact on the adjoining ovarian stroma. Thirdly, endothermic reactions, those that absorb rather than generate heat, may occur in maturing follicles and such a possibility needs to be explored systematically. Active chemical processes within Graafian follicles include synthesis and secretion of steroid hormones and likewise of diverse peptides, proteins and prostaglandins at changing concentrations. As ovulation approaches, there is remodelling of the tissues of the follicle wall, mucification of the cumulus oophorus, and resumption of meiosis in the relatively large primary oocyte (Dekel and Phillips, 1979; Thibault and Levasseur, 1979; Eppig, 1982). Constituent parts of these modifications might act to impose a degree of cooling, although heats of reaction for many of the presumptive processes remain to be defined.

The control experiment of removing and replacing an ovary after the initial temperature measurements was performed in part because there was a concern that infra-red sensing of the follicular surface might in some manner respond slightly differently to that of the stromal surface in living tissues, thereby generating artefacts. This line of thought may not stand up to rigorous inspection if it is recalled that the ovarian surface is composed of germinal epithelium, in reality the visceral peritoneum (Brambell, 1956; Baker, 1972a,b). Even so, the fact that the tissues of the excised ovaries showed uniform surface temperatures after initial chilling in liquid nitrogen and then rewarming in the abdominal cavity, with restoration of the original morphology, is informative in its own right. This observation strongly suggests that the temperature gradient between mature Graafian follicles and ovarian stroma depends, in part at least, on the activity of living tissues and integrity of the blood supply and lymphatic network. The precise components of tissue activity that might enable generation of the temperature differentials remain unknown, but the suggestion above of endothermic reactions in the follicle wall and/or fluid would now need to be taken seriously.

The infra-red technique measures surface temperatures rather than deep tissue temperatures, so there is a possibility that the present results may not give an accurate reflection of temperatures within the follicular antrum. However, after their initial rabbit study, Grinstead *et al.* (1985) measured intra-follicular temperatures in 25 follicles from 13 women and found that the antrum was indeed cooler than the ovarian stroma, sometimes by as much as 2.3°C. It is relevant to add that in parallel measurements on temperature gradients in the Fallopian tubes of animals in the present study, the infra-red estimates of surface temperature differences between the ampullary and isthmus portions were in line with those reported for corresponding portions of the lumen by Hunter and Nichol (1986). Using thermistor probes sited in the lumen, these authors found a mean temperature difference between rostral ampulla and caudal isthmus of 0.7°C in mated animals, the isthmus being cooler. Observations in the present series ($n = 4$) revealed a mean temperature difference in the same direction of ~1.0°C, but none of the animals had been mated. Moreover, the fact that temperature gradients can exist in other reproductive tissues deep within the abdomen strengthens the findings on ovarian temperature gradients.

One approach to the involvement of temperature in the normal progression of meiotic maturation in the oocyte would be to undertake in-vitro culture at temperatures lower than those currently used (e.g. 39°C; Cheng *et al.*, 1986) and note any anomalies in nuclear or cytoplasmic maturation or in the subsequent process of fertilization (Thibault, 1977). In particular, it would be valuable to examine by two-way gel electrophoresis whether the pattern of cytoplasmic proteins that appears during normal meiotic maturation (Moor and Warnes, 1978; Moor *et al.*, 1990) is perturbed during culture at, for example, 37.0°C or 38.0°C in comparison with the customary culture temperature used these days of 39°C. Although we are currently conducting such studies with the oocytes of domestic farm animals, principally those of cattle, it would also be instructive to examine the situation in human oocytes. As already discussed in Hunter (1990), a quantitative or temporal disruption in the protein constituents of the oocyte cytoplasm could provide one important explanation for the low incidence of normal embryonic development *in vivo* after transplantation of in-vitro matured and in-vitro fertilized oocytes. These remarks include specific damage to the oocyte cytoskeleton and microtubules of the meiotic spindle. It may well be that there are physiological temperature shifts during the processes of meiotic maturation and fertilization, and that a computerized programme with linear temperature shifts will be required to mimic these in in-vitro systems of culture.

Whilst it has seemed appropriate to focus on an influence of lowered temperature during the processes of oocyte maturation, consideration should also be given to an involvement of temperature in the actual shedding of the oocyte: the process of ovulation. Three specific points come to mind: (i) the proteolytic enzymes such as collagenase that act to depolymerize the wall of the follicle shortly before ovulation (reviewed by Thibault and Levasseur, 1979; Espey and Lipner, 1994) may function most incisively at values below that of deep-body temperature; (ii) the risk of haemorrhage from the vascular bed proliferating extensively through the cell layers of the follicle wall shortly before ovulation (Corner, 1919) may be reduced at lower temperature. Similarly, the process of blood clotting may be accelerated after discharge of the oocyte; (iii) the increasing coagulability of follicular fluid, observed especially in the pig as ovulation approaches (Hunter, 1984; Hunter and Poyser, 1985), may also be a physical reaction that is facilitated at a particular temperature below that of deep body temperature. Specific control of changes in coagulability with impending ovulation would seem essential, otherwise too viscous a follicular fluid could impede shedding of the oocyte into the Fallopian tube.

Finally, to broaden this discussion and consider gonadal strategies, diverse aspects of both ovarian and testicular function may depend critically upon a sensitive regulation of temperature, not least for reducing the risk of mutations in the germ cell line: the meiotic vulnerability of maturing germ cells has long been appreciated (Ehrenberg *et al.*, 1957). In males of a majority of eutherian mammals so far examined, gamete formation by the elaborate process of spermatogenesis requires the germinal epithelium to function at temperatures several degrees below that of abdominal temperature (Waites,

1970; Waites and Setchell, 1990). Failure of testes to descend through the inguinal canals into the scrotal sac, the so-called cryptorchid condition, is reflected in progressive destruction of the germinal epithelium (Griffiths, 1893; Crew, 1922; Moore, 1926) and is also associated with enhanced rates of testicular cancer in man. As to the ovary, the fact that it has retained a highly-coiled arterial blood supply reminiscent of that of a scrotal testis requires further consideration. Although this coiling may be primarily to facilitate arterio-venous exchange of hormones (McCracken *et al.*, 1971; Einer-Jensen, 1988), it could also contribute to regulation of temperature by impeding the rate of blood flow and by reducing the pulsatile nature of the arterial supply (T.D.Glover; personal communication). Overall, the coiling may make a significant contribution to successful ovarian function in the face of surrounding abdominal temperatures. It would therefore be instructive to apply techniques of vascular resection and anastomosis to examine ovarian function in the absence of highly coiled portions of the ovarian artery and vein (see Myren and Einer-Jensen, 1992). It would also be invaluable to monitor ovarian temperatures at different stages of the oestrous or menstrual cycle to note if there is a greater degree of temperature regulation during the follicular phase and with impending ovulation. This would best be done in conscious animals with previously-installed ovarian sensors to transmit information and with core temperatures recorded from deep arterial probes. Even though such an approach can never be viewed as 100% physiological, it would overcome concerns that anaesthesia may alter the partitioning of ovarian blood flow (R.M.Moor, personal communication).

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