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Temperature gradients in female reproductive tissues and their potential significance

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Abstract

This review challenges the long-standing dogma that deep-body temperature should be regarded as uniform. Not only may this not be so, but small gradients in temperature in and across reproductive tissues might have assumed functional importance during the evolution of eutherian mammals. Temperature gradients within the Fallopian tubes of estrous animals are interpreted in a context of pre-ovulatory storage of viable spermatozoa and their peri-ovulatory activation and release from the functional reservoir in the caudal isthmus. Proposals concerning the response of potentially-fertilizing spermatozoa to increasing temperature along the isthmus of the Fallopian tube are recalled, and application of the term *thermotaxis* to this phase of sperm migration is assessed critically. Classical findings on the temperature of Graafian follicles in rabbits and women are highlighted, and more recent work on temperatures in pig pre-ovulatory follicles is considered in detail. Although an experimental approach involving anaesthesia and infra-red sensing is open to criticism, the finding that pre-ovulatory follicles are cooler than ovarian stroma cannot be discounted as artifact. Instead, evidence for endothermic reactions that act to lower temperature within pre-ovulatory follicles is presented together with a description of relevant counter-current vascular physiology that enables maintenance of a cooler follicular temperature. As to future experimental work, the possibility is raised that temperatures may not be uniform across the cytoplasm of maturing oocytes nor at different stages of the cell cycle in very young embryos. These proposals lead to speculation that temperature may be exploited at a molecular level to modulate unfolding gene expression in zygotes and early cleavage stage embryos. Modern micro-imaging technology needs to be applied to such concepts.

Keywords: Fallopian tube, spermatozoa, thermotaxis, Graafian follicle, endothermic reaction, oocyte, zygote, gene expression, cloning.

Introduction

In the sphere of mammalian reproductive physiology, considerations of temperature have focused largely on the male (Harrison, 1975; Waites and Setchell, 1990). This is doubtless because of the scrotal location of the gonads in a majority of adult eutherians so far studied, reflecting a demonstrable susceptibility of the germ cell compartment to deep abdominal temperatures. Such susceptibility is highlighted in instances of cryptorchidism, but leads to the difficult question of explaining normal spermatogenesis in testicond mammals – those species in which the testes remain in the abdomen (Glover *et al.*, 1990). The precise temperature of intra-abdominal testes in an active state still requires clarification.

Despite such emphasis on the male, female mammals are known to show fluctuations in temperature according to the stage of the estrous or menstrual cycle. The changes have generally been monitored as rectal temperature in domestic animals and vaginal temperature in primates rather than in deeper tissues or organs of the reproductive system. Although there is a widespread notion that temperatures should be uniform within the abdominal cavity, this may not always be so. At the very least, short-term differences in temperature would be generated by the bulk intake of food and by drinking hot fluids or cold water. The contents of the gastro-intestinal tract could influence its surface film of peritoneal fluid and thereby the temperature of neighboring tissues. Gradients in temperature might also be demonstrable in deeper portions of the female genital tract and in the ovaries themselves (see below). Physiological mechanisms underlying such putative variations in temperature are the subject of current enquiry (eg., Luck *et al*., 2001; Ye *et al*., 2007). The practical significance of temperature variations is being considered in relation to optimizing *in vitro* systems for culture of oocytes, embryos and ovarian follicles (Ye *et al*., 2007).

Earlier work of the author and his colleagues has been presented as original papers (Hunter and Nichol, 1986; Hunter *et al*., 1997, 2000) and in more recent reviews (Hunter, 2003; Hunter *et al.*, 2006). The sections that follow introduce a number of new considerations and offer constructive criticism of earlier work. Further measurements in animals held under controlled conditions are not yet available.

Temperature gradients in the Fallopian tubes; progression of spermatozoa

In sheep, pigs and cows mated in the early part of estrus, viable spermatozoa are arrested and stored before ovulation in the caudal (distal) portion of the Fallopian tube. This region is termed the *functional sperm reservoir*. Spermatozoa suspended in uterine

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fluids shortly after mating are highly active but, during the pre-ovulatory phase, such motility is dramatically reduced upon passage through the utero-tubal junction into the viscous milieu of the caudal isthmus. In this location, the heads of viable spermatozoa undergo specific epithelial binding to either cilia or microvilli or to both (Fléchon and Hunter, 1981; Hunter *et al*., 1987, 1991).

Fluid in the tightly-constricted lumen of the isthmus represents predominantly accumulated glycoprotein secretion; its viscosity and volume are regulated by the stage of the estrous cycle relative to ovulation. Shortly before actual ovulation, and with the switch of steroid synthesis in mature Graafian follicles from estradiol to progesterone, there are changes in sperm interactions with the endosalpinx. Spermatozoa are gradually released from epithelial binding and, undergoing progressive hyperactivation, proceed along the isthmus to the site of fertilization. Initially, the number of viable spermatozoa at the isthmo-ampullary junction is low and initial sperm:egg ratios may be close to unity. However, with time elapsing after ovulation, and especially with enhanced secretion of ovarian progesterone, progressively more spermatozoa are activated and released from the caudal isthmus and large numbers of spermatozoa may confront eggs at the site of fertilization. Under a normal sequence of events such increased numbers of spermatozoa do not prompt abnormalities of fertilization since penetrated eggs would already have established a stable block to polyspermy. Instead, accessory or supplementary spermatozoa accumulate in the zona pellucida of recently-fertilized eggs and may achieve numbers of 200-400, for example, in individual pig zygotes.

The above perspective has been given by the author in many reviews, most recently in Hunter (2008). Studies on mechanisms of sperm binding in the isthmus have also been prominent in recent years, with clarification of the molecules involved on both the sperm head surface and the apposing endosalpinx (Smith, 1998; Suarez, 1998, 2001; Talevi and Gualtieri, 2001; Töpfer-Petersen *et al*., 2002). Such studies invariably emphasize the role of sperm head binding in creating a functional sperm reservoir in the isthmus, but they seldom appreciate or reflect on the underlying physiology, yet the Fallopian tubes are not quiescent structures. During estrus, in particular, they are tonic, edematous, and showing powerful contractile activity. Myosalpingeal activity is enhanced even further in the presence of mature males, and dramatically so upon mounting, intromission and semen deposition. Were it not for pre-ovulatory binding of spermatozoa in the caudal portion of the isthmus, large numbers of spermatozoa would already be present at the site of fertilization by the time of ovulation. This situation would result from powerful ad-ovarian waves of myosalpingeal contraction giving bulk displacement of viable spermatozoa. Polyspermic penetration of oocytes would be an inevitable and disastrous sequel.

Using this description of pre-ovulatory sperm storage in the caudal isthmus and controlled peri-ovulatory activation and release as background, the question arose as to whether some long-standing measurements of temperature in the Fallopian tubes could have relevance (David *et al.*, 1972). In particular, might local changes in temperature be associated with the phases of sperm storage and release? Accordingly, temperatures were measured in pig Fallopian tubes early in estrus and shortly after ovulation, approximately 36-40 hours later (Hunter and Nichol, 1986). Mid-ventral laparotomies were performed under general anesthesia, and fine thermistor probes introduced into opposing ends of the same Fallopian tube. One probe was sited in the caudal isthmus, having entered through the utero-tubal junction. The second probe was introduced via the fimbriated extremity and sited in the proximal (cranial) portion of the ampulla. Both probes were connected to the same temperature scale and were calibrated before each set of measurements and accurate to 0.1^{o}C. The incision was closed, and temperatures recorded after a period of stabilization.

In a series of 10 mated animals examined early in estrus, the caudal isthmus was cooler than cranial ampulla by a mean of 0.7^{o}C (range 0.2-1.6^{o}C). In a further series of 12 animals examined shortly after ovulation, the mean temperature difference between the two ends of the Fallopian tube was 0.1^{o}C. The pre-ovulatory temperature differences were thought primarily to reflect the extent and activity of the vascular and lymphatic beds and of myosalpingeal contractions and, together with specific chemical microenvironments, would facilitate the relatively prolonged period of sperm storage in the caudal isthmus (Hunter and Nichol, 1986). Not appreciated at the time was the fact that endothermic reactions could be generated in the viscous glycoprotein secretions that accumulate in the caudal isthmus before ovulation. During and after ovulation, an increase of temperature in the storage region would facilitate activation and release of maturing spermatozoa.

Further contributions on sperm progression in the Fallopian tube

A publication in *Nature Medicine* in 2003 contained the proposition that thermotaxis guided movement of a competent spermatozoon towards the site of fertilization (Bahat *et al*., 2003). Two subsequent papers from the same group developed the proposition that thermotaxis and chemotaxis worked sequentially in the preliminaries to mammalian fertilization (Bahat and Eisenbach, 2006; Eisenbach and Giojalas, 2006). However, the contention that sperm thermotaxis contributed to the events leading up to mammalian fertilization seemingly missed a simple alternative explanation for the orientation of sperm swimming

activity. In addition, the authors overlooked a considerable body of evidence that undermined their portrayal of events.

The original observations in Hunter and Nichol (1986) were developed in two subsequent reviews (Hunter, 1997, 1998), especially after further detailed studies of temperature gradients in pre-ovulatory mammalian ovaries (see below). Temperature physiology appeared to be involved in the final maturation of both male and female gametes. One of our proposals was that during the phase of peri-ovulatory sperm activation from the isthmus reservoir and during completion of the final stages of capacitation, spermatozoa may be programmed to swim up a temperature gradient to the site of fertilization at the ampullary-isthmic junction (Hunter, 1997). The most exciting thought here (adjective used physiologically) was whether … "competent spermatozoa – those with potential fertilizing ability – are finally heat-seeking cells, and could this not be a vital functional context in which to view temperature gradients within the Fallopian tubes?" (Hunter, 1997). The membranous state of spermatozoa activated close to the time of ovulation in the isthmus reservoir may have rendered them responsive to small gradients in temperature. Temperature rather than chemotaxis may act significantly in eutherian mammals to influence the efficiency of sperm-egg interactions (Hunter, 1998).

Such considerations concerning sperm progression and temperature therefore have a clear origin, but the word *thermotaxis* was specifically avoided in our publications. Although we wrote about potential heat-seeking systems on the sperm head being revealed during the process of capacitation, spermatozoa may not be *attracted* to a region of warmer temperature. Rather, their progression within the Fallopian tube may simply reflect increased sperm motility generated by an increase of temperature. In other words, appropriate sperm cells may be propelled by enhanced flagellar beat as a consequence of increased temperature and metabolic activity rather than due to a system of heat detection on the anterior portion of the sperm head with transduction of information to the mid-piece and flagellum. A system of thermotaxis is certainly not proven.

Evidence against the notion of thermotaxis can be drawn from diverse experimental observations and indeed from straightforward physiological considerations. As to experimental evidence:

a) instillation of an appropriate sperm suspension *via* the fimbriated infundibulum into the ampulla can lead to normal fertilization in rabbits, sheep and pigs (eg., Chang, 1951; Mattner, 1963);
b) intra-peritoneal insemination can result in fertilization and pregnancy in a range of mammalian species (Yaniz *et al*., 2002), and in retrograde sperm transport to the uterus and cervix (López-Gatius and Yaniz, 2000);
c) ad-ovarian transport of particulate matter or boluses of oil to the site of fertilization close to the time of ovulation is associated with progression of cilial beat and myosalpingeal contractions (Blandau and Gaddum-Rosse, 1974; Battalia and Yanagimachi, 1979). Such peri-ovulatory activities would override any putative influence of thermotaxis.
d) after spontaneous mating, spermatozoa pass from a warmer uterine lumen to a cooler caudal isthmus during the pre-ovulatory interval. Although incomplete capacitation may render spermatozoa insensitive to small temperature gradients at this stage, such contrary evidence nonetheless requires consideration.

In the light of this range of observations, caution is urged in any repetition of notions of thermotaxis being a significant feature in the preliminaries to mammalian fertilization. In summary, there is a duty to produce rigorous new evidence.

Temperature differences across ovarian tissues

Once again, there are well-established observations on temperatures in mammalian ovaries. Studies in Copenhagen found that pre-ovulatory follicles in rabbits were 1-2^{o}C cooler than ovarian stroma; infra-red and microelectrode measurements were made at laparotomy (Grinsted *et al*., 1980). In women, Graafian follicles were as much as 2.3^{o}C cooler than ovarian stroma as determined with thermoelectrodes sited in the follicular antrum (Grinsted *et al*., 1985). In our own preliminary studies in Edinburgh, pre-ovulatory pig follicles of 9-10 mm in diameter were >1.0^{o}C cooler than ovarian stroma. These results were obtained using thermistor probes introduced into the follicular antrum, but were not submitted for publication due to the scope for artifacts. Puncture of the follicle wall and consequent modifications to the vascular bed were of major concern.

However, an opportunity arose in 1995 to resume such studies in pigs at the Royal Veterinary University in Copenhagen. Large Graafian follicles (7-10 mm in diameter) were sensed by infra-red technology during spontaneous estrous cycles (Fig. 1). Observations in 14 animals during mid-ventral laparotomy revealed that ovaries were always cooler than deep rectal temperature, and that mature follicles were always cooler than ovarian stroma (35.6 $\pm$ 0.3^{o}C versus 37.3 $\pm$ 0.2^{o}C, respectively; $P < 0.01$). Such follicles were frequently 1.5-1.8^{o}C cooler than adjacent stroma, whereas small Graafian follicles (<5-6 mm in diameter) and recent ovulations did not show this differential (Hunter *et al*., 1997). Using a later model of infra-red camera, these thermosensing measurements were extended in a series of 73 observations on 7-10 mm in diameter Graafian follicles (Fig. 2); these were a mean of 1.3 $\pm$ 0.1^{o}C cooler than neighboring ovarian stroma. There were no exceptions to the finding that mature follicles were always cooler than stroma (Hunter *et al*., 2000).

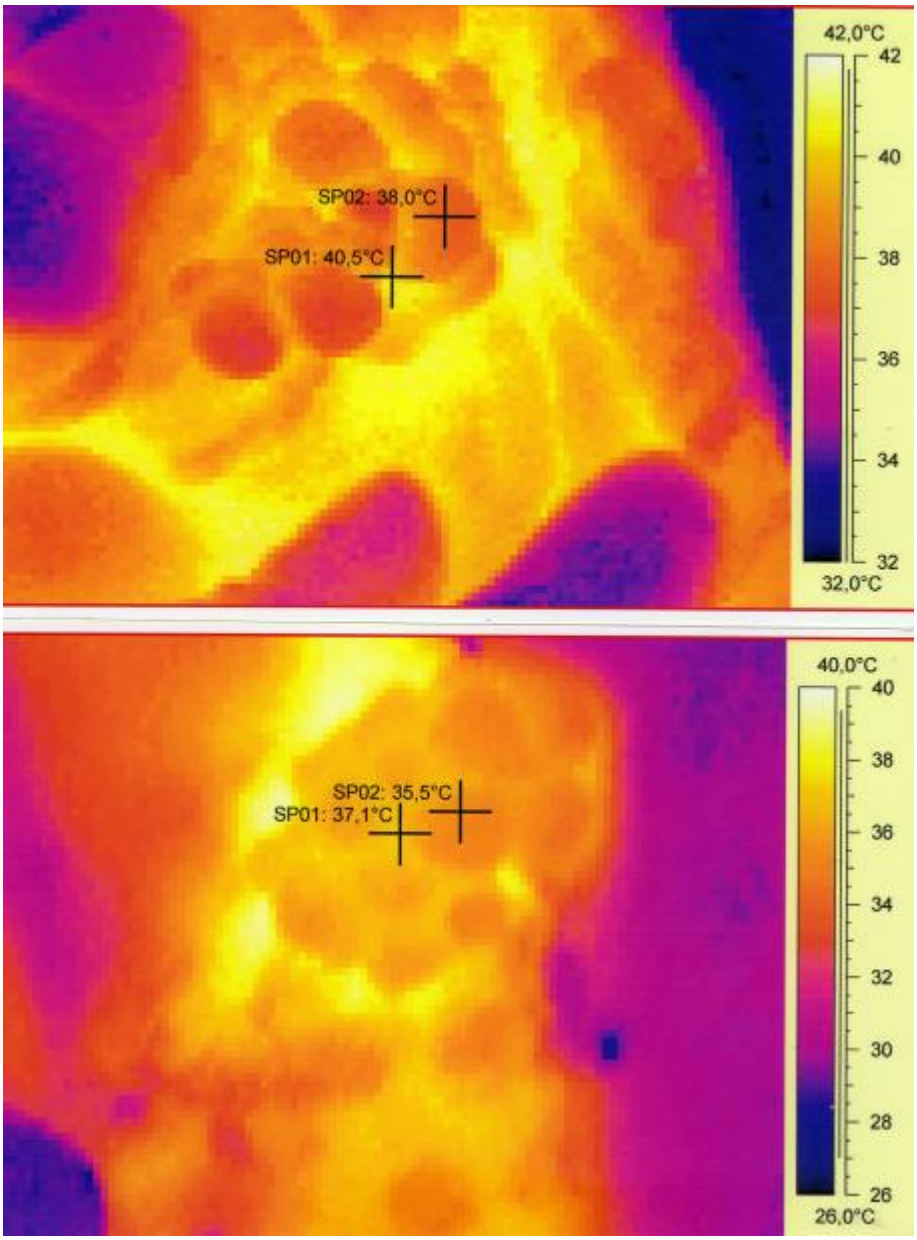

Figure 1. Infra-red images of pre-ovulatory pig ovaries demonstrating that large Graafian follicles (7-10 mm in diameter; SP02) are cooler than neighboring ovarian tissue (SP01) and the surrounding peritoneal fluid.

Although there was a minor concern that measurements were made during mid-ventral laparotomy, the infra-red camera was pre-positioned above the site of incision, recording of temperature was almost instantaneous, and the operating room was pre-warmed to 28-30°C. Confidence in the observed temperature differences came from the fact that follicles and stroma could still be distinguished when the ovary was thermoimaged under the fimbriated extremity of the Fallopian tube. Furthermore, cooling curves for the exposed tissues examined during a 60 sec interval were effectively in parallel for mature follicles and stroma (Fig. 3). Endoscopy of ovarian tissues in three animals revealed differences between follicles and stroma of 0.6 $\pm$ 0.1°C to 1.1 $\pm$ 0.1°C, but the Graafian follicles in all three animals were predominantly of 6-7 mm in diameter and not yet of pre-ovulatory size (Hunter *et al*., 2000).

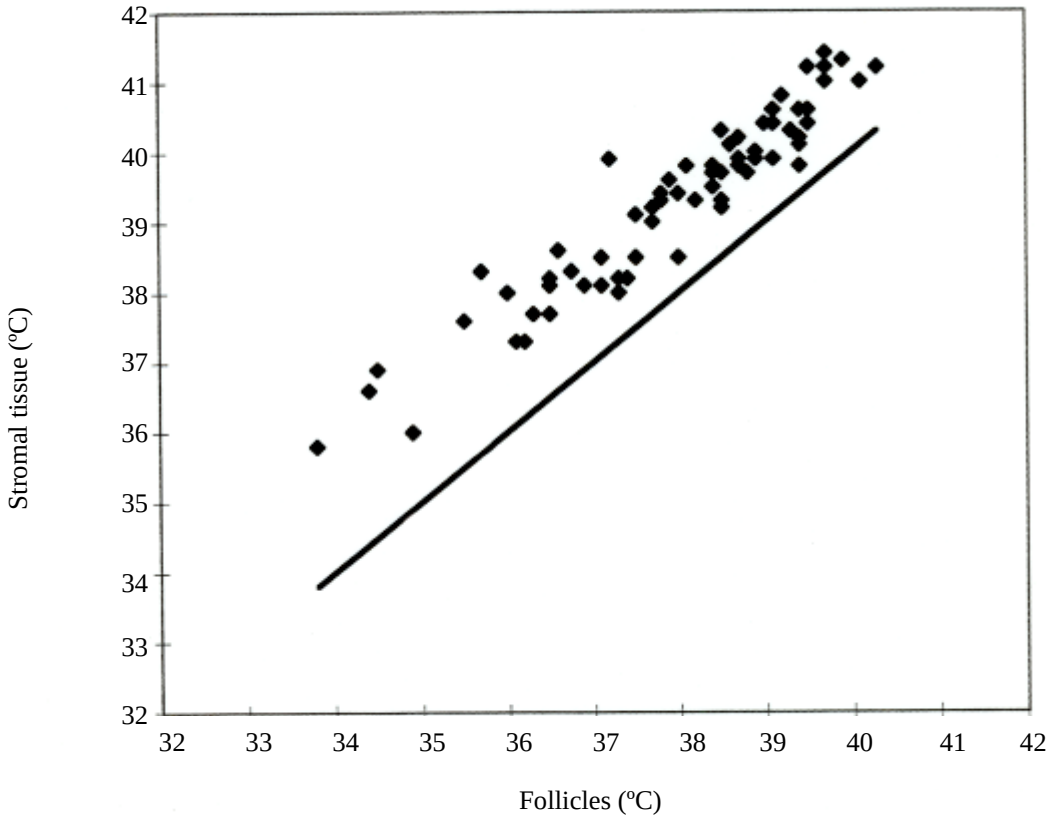

Figure 2. To portray the consistent finding that pig Graafian follicles (7-10 mm in diameter) were always cooler than adjoining ovarian stroma as determined by thermo-imaging. The graph summarizes 73 sets of observations in which the straight line would represent temperature equality between the two tissues.

Establishment and maintenance of intra-ovarian temperature gradients

Assuming that artifacts were not a primary cause of the observed temperature differentials, then endothermic reactions within mature Graafian follicles may have been a contributory factor. Physico-chemical evidence for such reactions within the components of follicular fluid is available from *in vitro* observations. Saline dilution of bovine follicular fluid in a special incubator could depress temperature by 0.14-0.2^{o}C. This drop in undated *post mortem* samples of unfractionated fluid (*not* specifically pre-ovulatory) was sustained for 7-13 min (Luck and Griffiths, 1998; Luck *et al*., 1999). There is also provisional evidence that hydration of large molecular weight constituents of follicular fluid can act to depress temperature (Luck and Gregson, 2000; Luck *et al*., 2001). Bearing in mind the extensive mucification of the cumulus oophorus that occurs in pre-ovulatory follicles, then hydration of large molecules such as proteoglycans may have contributed significantly to the observed cooling of follicles (Hunter, 2003).

A means for the maintenance of intra-ovarian temperature gradients also needs to be considered, since systemic blood circulation might be expected to override any influence of local cooling mechanisms. In particular, there would need to be a counter-current exchange of heat in the blood supply to and from individual Graafian follicles (Fig. 4). Details of the relevant ovarian vasculature, including the microvasculature of follicles, and scope for counter-current exchange are given in Hunter (2003), Einer-Jensen and Hunter (2005), and Hunter *et al*., (2006). The anatomical basis and physiological potential both exist to facilitate a local maintenance of temperature gradients. Moving from individual follicles to the whole ovary, counter-current exchange systems are well-established in the gonadal 'pedicle' of male and female mammals, and their involvement in the present context is reviewed in Hunter *et al*. (2006). This includes a consideration of the efficiency of ovarian heat exchange mechanisms.

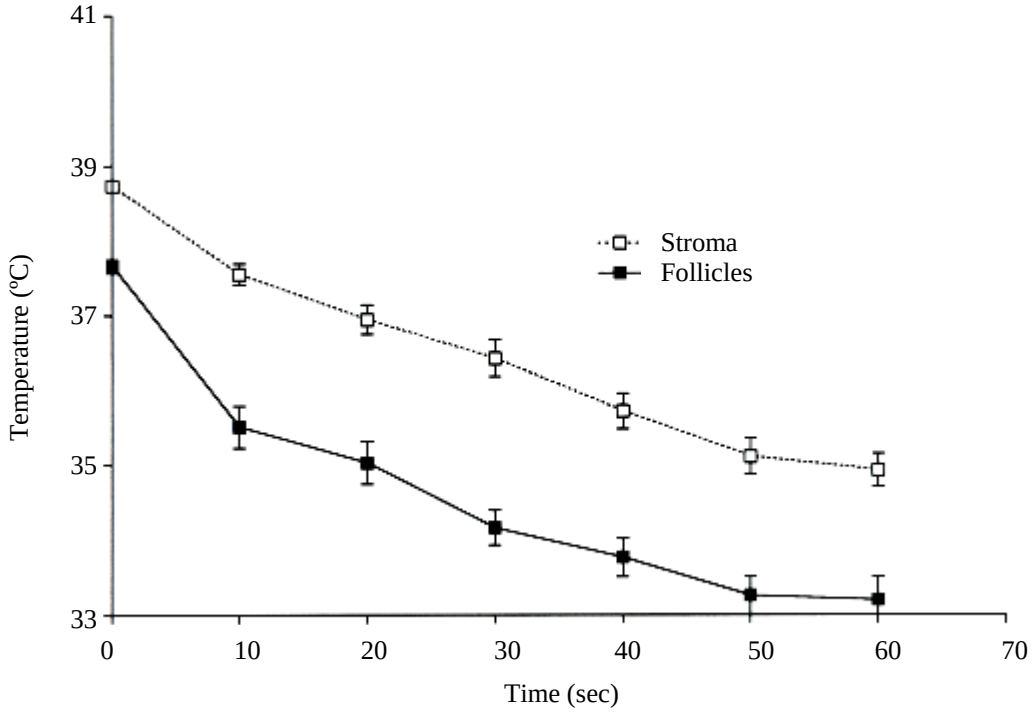

Figure 3. Curves of tissue cooling rates during a period of 60 sec when pig ovaries were exposed at mid-ventral laparotomy. The curves were essentially parallel for ovarian stroma and pre-ovulatory Graafian follicles, as determined by thermo-imaging.

Physiological and molecular significance of temperature differentials

Far from regarding deep abdominal temperatures in female mammals as uniform and constant, it is time to consider the extent to which the evolution of systems of internal fertilization and embryonic development has presented opportunities for subtle regulation of reproductive processes by temperature gradients. Changes in follicular temperature might be critically involved in the events of ovulation, for example by modifying the activity of proteolytic enzymes needed to depolymerize the follicle wall, the distensibility and dissociation of tissues, remodelling of the basement membrane, and the coagulability of follicular fluid (Hunter, 2003). Capillary blood flow and the extent of hemorrhage in the collapsed follicle wall could certainly be modified by local changes in temperature, and likewise the mobilization and infiltration of polymorphonuclear leukocytes. The biosynthesis of gonadal steroid hormones would be sensitive to temperature regulation.

At a cellular and sub-cellular level, nature may have exploited gradients in temperature for regulating the physiological activity of gametes and embryos. Some reference to the influences of temperature on spermatozoa has already been made, with motility and membranous modification as primary considerations. As to oocytes and zygotes, changes in temperature could influence the activity of both nucleus and cytoplasm and the characteristics of nuclear and cell membranes, including the remodelling of structural proteins. Small deviations from physiological temperature could have a profound influence on subsequent patterns of gene expression. For example, the prompting of gene cascades in the apoptotic pathway during *in vitro* maturation of oocytes at inappropriate temperatures or other forms of perturbed gene expression (McEvoy *et al.*, 2003; Ye *et al.*, 2007) endorse an involvement of temperature in the regulation of gene expression. The precise stage(s) of waywardness in the transcription-translation sequence remains to be revealed. Likewise needing further consideration are the role and full significance of heat shock proteins, not to mention the various consequences of disease and hyperthermia.

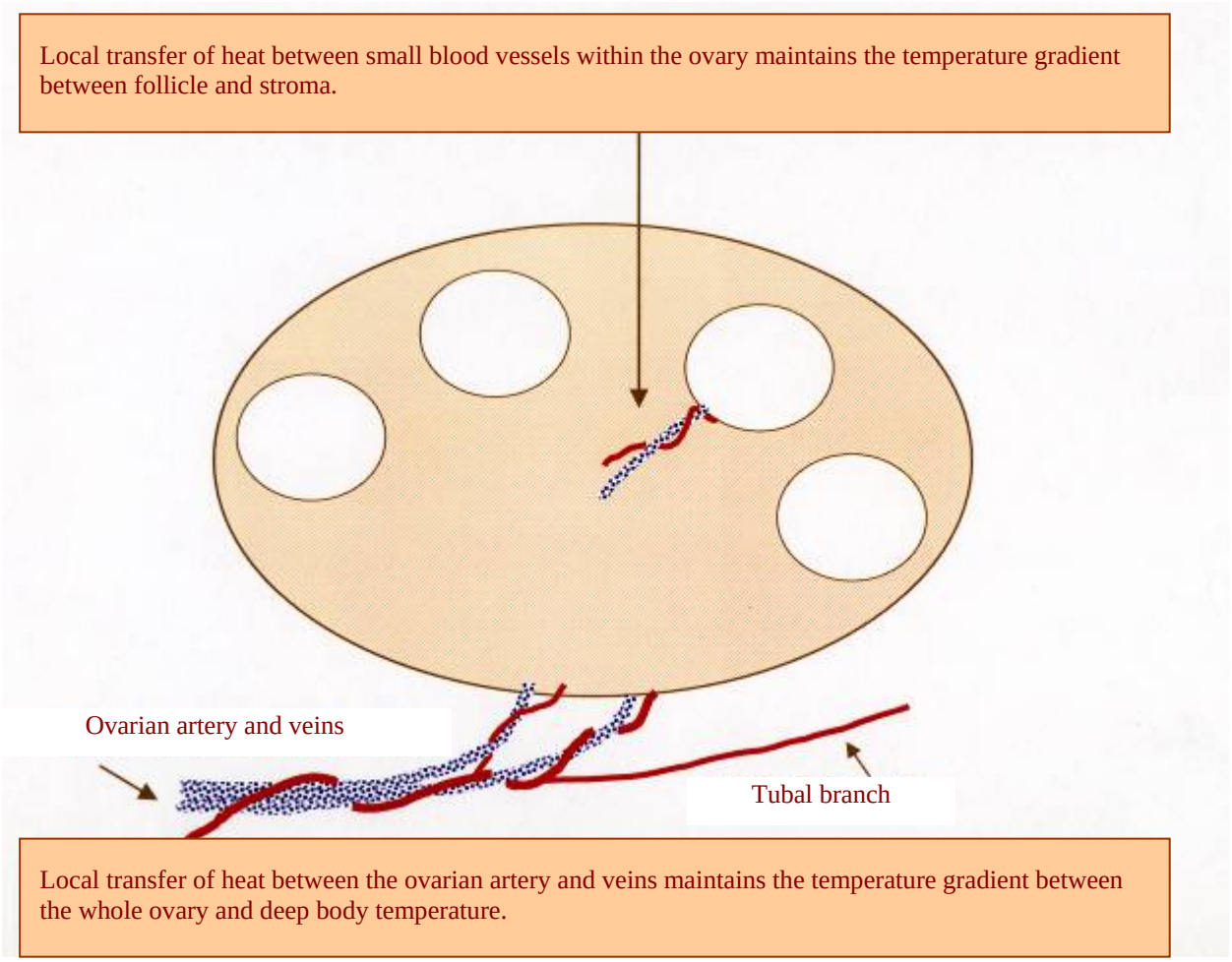

Figure 4. Diagrammatic representation of an ovary with large Graafian follicles to illustrate the pattern of vasculature which would permit a counter-current exchange of heat both in the ovarian pedicle and close to mature Graafian follicles.

The future: novel imaging technology

There is much speculation in the above but the way is open to appropriate experimentation. Modern micro-imaging techniques should enable examination of temperature changes in oocytes and embryos in culture. Of particular interest is whether there are subtle changes in temperature during the cell cycle and indeed whether gradients in temperature exist within and across a cell, influenced not least by cell size during the first cleavage divisions as expression of the embryonic genome commences. If there is any validity in these ideas, then exploring the effect of temperature shifts during culture of oocytes might be one approach to modifying nuclear-cytoplasmic interactions. In other words, exposing enucleated oocytes to a precisely-regulated gradient in temperatures at the time of introducing somatic cell nuclei might facilitate their remodelling and reprogramming and be a fruitful way forward in techniques of mammalian cloning.

At the whole body level, non-invasive imaging technology that can reveal deep body temperatures at precise locations and to an accuracy of $\pm$ 0.1^{o}C would be invaluable for determining the extent of physiological gradients in the reproductive tissues of fully-conscious animals. Such measurements at different stages of the estrous or menstrual cycle might then be usefully extended to immediate pre- and post-coital situations and to animals receiving hormone stimulation or undergoing treatment for synchronization of estrous cycles. And, as a not completely unrelated point, differences in temperature associated with differences in peritoneal fluid composition would be of especial interest. The nature of regional fluid environments within the peritoneal cavity is a developing topic of research (Hunter *et al*., 2007; Cicinelli *et al*., 2008).

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