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Review Article

The Impact of Testicular Hyperthermia and Its Physiological Relevance to Human and Agriculture

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Abstract

Testicular heat stress is a well-known and well-described phenomenon that occurs in mammals that possess a scrotum. Different models to induce testicular hyperthermia, such as surgical cryptorchidism, hot water bath, scrotal insulation or increased environmental temperature have all shown that spermatocytes and spermatids are unambiguously affected by high temperature, resulting in poor sperm production weeks later. The testis appears to be very sensitive to temperature fluctuations, as even small changes in scrotal temperatures cause a drop in sperm counts, motility, and morphology. The higher the temperature and longer the during of heat, the more pronounced the effect in terms of semen quality. Whilst "experimental" models seem to validate the effect of testicular heat stress on sperm quality, the physiological relevance of testicular hyperthermia is still debated. Herein we summarise a cohort of studies that report the effect of "season" on sperm quality. The data show season can affect semen quality depending on where the work was performed. In countries where temperatures drop below zero, there is evidence to show that summer conditions tend to improve semen quality. However, in sup-tropical countries, there is some debate; some studies showing decreases in summer, whilst others show no change. Herein we offer an explanation as to explain this apparent discrepancy. Furthermore, we present data that show individual animals can show a "spectrum" of tolerance when it comes to spermatogenesis and testicular hyperthermia.

Keywords: Testicular heat stress, agriculture, bulls, thermosensitivity

The Different Models of Testicular Hyperthermia all Show Similar Outcomes.

The impact of testicular heat-stress has its origins back in 1893, when Joseph Griffiths noticed nobody had tried to "experimentally determine" the origins of "John Hunters" observation that testicles failed to reach their full size and produce sperm when retained in the abdomen (cited in 1). Griffiths was interested to understand if the lack of testis growth was the fault of a "young-testis", being imperfectly formed at the beginning then failing to descend and mature, or whether a testis that has reached its "full and mature size, is capable of maintaining that full size and its powers of producing spermatozoa" (cited in 1) when placed back into the abdomen. By performing unilateral surgical cryptorchidism in Fox Terriers, Griffiths noted the testis "dwindle to a considerable extent" and are incapable of producing spermatozoa after 6 months [1]. Unbeknownst at that time, this work heralded the beginning of the field of testicular hyperthermia. Since then, a diverse range of experimental models have been used, all of which confirm that spermatogenesis is a heat-sensitive process.

Such testicular hyperthermia models seek to do one of two things; either prevent the scrotum from descending or overwhelm the ability to scrotum to regulate testicular temperature. With regards to the latter, surgical cryptorchid models or wrapping the scrotum up in woolen cloths and holding the testis close to the abdomen to prevent its descent has been performed in rabbits [2], dogs [3], rams [4,-6] bulls [7-15] and boars [16]. In all cases, a decline in sperm concentration, motility and morphology was shown. In the case of bulls (one of the most-studied model species,) a decline in semen quality begins 5-15 days post-intervention, peaking around 4 weeks later [7-15].

The use of a hot-water bath [17-27] and elevated air temperatures [28,29] are examples of two models used to "overwhelm" the ability of the scrotum to regulate heat. The advantage of these models is the ability to test a range of temperatures and their impact on sperm production. In the case of the rabbit [29], ram [28] and boar [16], increasing the ambient air temperature even for 1 hour, showed a significant decline in sperm parameters, particularly motility [29]. When left for longer (up to one week, 32°C), a significant decline in all sperm parameters (motility, morphology and counts) can be seen 5 weeks later. Notably, semen quality beings to improve 8 weeks post intervention, suggesting spermatogonia are not affect by short-term heat duration.

In terms of how different temperatures affect spermatogenesis, the best documented models come from using hot-water baths on anesthetised mice. From this work, it is very clear that either the higher the temperature, or longer the duration of time spent in the water bath, the more detrimental this is to spermatogenesis. For example, scrotal heating, with the water bath temperature set to 41°C, 42°C or 43°C for 30 minutes reduces sperm counts by 54%, 76% and 88% respectively [21]. Furthermore, mice that receive higher degree of testicular hypothermia take longer to recover [21].

How Hot is Too Hot for the Testis?

To answer this, early measurements of testicular temperature were done in the rat, rabbit and guineapig [30]. By comparing the "core" body temperature of these animals, to that of the internal testis temperature, seemingly large differences were found. For example, when the environmental temperature was set to 16°C, the difference in scrotal vs peritoneal temperature was up to 8°C. Yet raising the environmental temperature to 32°C saw this difference reduce to just 4°C [30]. Notably, modern measurements in rats housed at 22°C, suggest the testis sit 3.5°C lower than core body temperature [31]. Recent use of mouse organoid cultures [32] appears to help shed light on this question. Testicular explants cultured at 34°C are able to grow and enlarge over 5 weeks. However, by raising the temperature in 1°C increments from 30-40°C, changes in spermatogenesis can be observed. Whilst 34°C resulted in optimal spermatogenesis progression, germ cell development sill occurs at temperatures between 32-35°C. However, at 36°C, elongating spermatids disappear from the culture, with only a few round spermatids present, suggesting no progression following the pachytene stage. When cultured at 37-38°C, only spermatocytes can be detected, which appeared to be a result of meiotic prophase I failure at the pachytene and diplotene stages especially. These same temperatures also induced DNA double strand breaks, which ties in with many physiological models that show heat-stress leads to increased DNA damage [33]. Put together, these data suggest, at least the optimum temperature in the mouse is around 34°C. One degree either side of this is permissible, but once the temperatures exceed 35°C, spermatogenesis is largely affected.

Is "Heat-Stress "Physiologically Relevant

The use of testicular-hypermetria animals models make it easier to study the impact of heat on sperm quality. However, the question remains as to whether this is a physiologically relevant? especially in agriculture species and humans. The first report of seasonal effects on breeding efficiency in dairy cattle occurred in 1938[34]. Dairy records taken from the University of Nebraska showed more "services" were required for conception from May to October (i.e. summer) compared to winter months. This appeared to be consistent throughout the years 1896 to 1934 [34] . In 1941, an examination of the eleven-year reproductive history of breeding records of the Lousiana State University showed seasonal differences in the rate of conception [35]. Again, summer months required more services per conception, whilst the best record for rate of conception was during winter months [35]. These data pointed to two possible interpretations. Either the cow, or the bull were somehow affected by heat stress. The notion that physiological temperatures could affect bull fertility was then advanced in 1942, when Purdue University reported that on average, semen volume and motility were least in July, August and September (summer) whilst the average concentration was maximum during April, May and Juner [36]. Finally, Anderson showed bull sperm volume and motility declined during summer in Kenya [37]. Experimentally, the idea that summer could be causing a drop in semen quality this was then verified by the purposeful heating of dairy bulls inside chambers at physiologically relevant (32.2°C) temperatures. Such bulls showed clear evidence that a warm environment can lead to a decrease in sperm morphology and motility [38].

Despite these early works, the impact of "season" on bull fertility does not come without controversy. Reports from Cornell University, show winter was the poorest season for breeding dairy cattle in Canada and New York State [37,39] which has since been confirmed by others [40-43]. In deference others report that there is seasonel effect on bull semen quality [40,44-46]. In order to make sense of this, we have taken a closer examination of some studies

which look at the effect of "Season" on bull semen parameters (Table 1).

In table 1, where available, the motiltiy, morphology and cell counts from each study are included. If the study reported the average temperatures at the time the work was perfromed, this was also included, otherwise the data was obtained online (weather-and-climate.com) using seasonal averages. Altough this table does not list all the works perfromed in this area, it is indeed very indicative of the results in this field. From this work, it is quite evident there are two variables that need to be considered, both of which appear to explain much of the "contradictory" results around "season" and "semen quality".

Firstly, most of the work in Table 1 is perfromed at very different locations, with extremly different climatic conditions. For example, in both Kenya and Thailand in which ambient temperature is consistent thoughout the year, little variation in semen quality was observed [44,51] as expected. However, in USA [45] and Sweden [68] where winter temperatures drop below zero, it is little surprise that sperm motilty is higher during summer.

The most controversal work comes from countries considered sub-tropical, for example in Brazil and Austrlia. Herein, summer temperatures can exceed 40^{o}C, whilst winter temperatures can drop down to 10^{o}C. Based on the experimental work (purposeful heating in a chaber, ater bath etc), it would be expected that such temperatures would impact bulls, if season played a role. Indeed, there are reports in these countires and clear evidence of semen quality deteriorating in summer [60]**.** However, this is not always the case with one major report from Australia which looked at 11,387 bulls and found to be no evidence of seasonal variation [69]. In this report, bulls were sourced from all over the counter (as opposed to one area). As Australia is so large, the changes in temperature from the north to south can be so vast it makes it difficult to analyse. However, these same authors did note that bulls found in the hottest part of the country (north region) were less likley to pass a semen test. Yet in our own results, we find definative evidence of "seasonal" variation within bulls taken from central QLD, Australia (REF). How then do we account for the "apparent" contradiction. The answer to this comes down to the second variable and how the bulls were analysed. Our work shows within a co-hort of bulls, there were large degrees of heat-sensitivity (outlined below). Some bulls going "off" during the summer heat, whilst others being totally unaffected. If all bulls are analysed as a "co-hort" one finds no statistical difference in semen quality and season due to the large variation of animals that do and do not respond. However, when this is accounted for, it is clear that summer conditions lead to a definate change in semen quality (REF).

# bulls	Type	# ejaculates	Motility (%)		Cell counts		Morphology (% defective)		Country	Ave, temp (^{o}C)		Ave Humidity (%)		Ref
			Sum.	Win.	Sum.	Win.	Sum.	Win.		Sum.	Win.	Sum.	Win.	
6	B. Indicus	n/a	55	39	1.2^{9}	1.1^{9}	39	30	Khon kaen, Thailand	36	30	80	63	[42]
51*	Bos Indicus/ indicus	ND	NSD**	NSD**	11^{9}	11^{9}	14	10	Sao Paulo, Brazil	~26	~19	84	51	[44]
13	Bos Taurus	1103	4.2***	3-3.7**	1023/ mm3	1015/ mm3	14	14	Missouri, USA	24	-3	60	65	[41]
9 bulls (reduced to 2 over time)	Zebu	1049	75	80	600cc3	700cc3	ND	ND	Kenya	27	20	75	60	[47]
137	Bos Taurus	5644	55	56	13.5-14^{9}	14.5-15.5^{9}	ND	ND	Geuth, Canada	26	-3	60	65	[40]
5	Swamp Buffalo	118	73	75	4.2^{8}	3.6^{8}	10	11	Khon-Khaen, Thailand.	35	33	89	92	[45]
27	16 Bos. Taurus. 11 Bos. Indicus	ND	NSD	NSD	ND	ND	20	12	Dourados, Mato Grosso do Sul, Brazil	29	22	80	75	[48]

10	Bos Taurus	ND	47	55	ND	ND	14	15	Uppsala, Sweden	18	-3	65	94	[49]
5	Bos Taurus	86	40	49	ND	ND	ND	ND	Hafetz-Haim, Israel	31	16	84	46	[50]
52	Bos Taurus	86	ND	ND	ND	ND	12	11	Sweeden	21	17	74	40	[51]
10	Bos Taurus	ND	58	57	1.8^{9}	1.7^{9}	39	27	Zamiba	28	8	45	59	[46]
218	Bos Indicus	ND	68	70	6.6^{8}	5.7^{8}	ND	ND	Brooksville, Florida, USA	32	19	79	73	[43]
11	Bos Taurus	ND	58	51	ND	ND	27	12	Gijon, Span	21	11	75	75	[52]
7	Bos Indicus	142	28%	36.2	4.1^{8}	3.9^{8}	21	30	Nsukka, Nigernia	27	24	83	44	[53]
2	Buffalo bulls	42	ND	ND	ND	ND	27	18	Pantnagar India,	36	22	28	45	[54]
2	Ongole	86	56	55	8.2^{9}	8.5^{9}	ND	ND	Semarang, Indonesia	27	27	81	82	[55]
	Simmental	89	70	70	9^{9}	9.7^{9}	ND	ND						
271	Bos Taurus	ND	ND	ND	ND	ND	27	17	Irene, South Africa	28	20	60	37	[56]
19	Bos Taurus	ND	ND	ND	ND	ND	14	11	Northern USA	?	?	21 to 43	5 to -30	[57]
6	5 ejaculates per season	ND	70	70	1.4^{9}	1.6^{9}	9	7	30	21	8	85	84	[58]
21	Bos Taurus	ND	51.5	54.6	ND	ND	14	15	Spain/ Sweden	18	2	78	88	[59]
11	Bos Indicus /Taurus	2558 (1095 *B. indicus*	57	58	1.6^{6}	1.4^{6}	27	16	S.E. Brazil	25	19	83	72	[60]
		1463 *B. taurus*)	51	59	1.2^{6}	1.2^{6}	44	18						
933	Bos Taurus	29170	90	84	ND	ND	3	7	Netherlands	15	10	95	62	[61]
176	Bos Taurus	8983	82	82	9^{9}	~9^{9}	ND	ND	Ireland	14	6	83	68	[62]
3	*Bubalus bubalis*	ND	65	64	5.2^{9}	3.4^{9}	ND	ND	Indonesia	23	23	79	74	[63]
155	*Bos sondaicus*	155	ND	ND	12^{6}	14^{6}	ND	ND	Townsville, Australia	33	13	76	64	[64]
288 abattoir/21 breeding bulls	Bison	ND	69	44	7.1^{8}	5.1^{8}	39	43	Alberta,			10	0	[65]

7	*Bubalus bubalis*	4834	66	68	1.16^{9}	1.11^{6}	ND	ND	Salon, India	37	25	62	55	[66]
8	Bos. Taurus and crossbred	558	79	81	3.31^{9}	10.1^{9}	24	5	Nigeria	37	27	87	76	[67]
* Three different collections stations used; Reported as no significant difference, but no values given. Motility scored from 0-5 (with 5 being the highest). Using data from 48-59 months.														

Table 1: A snapshot of studies that have looked at the effect of "season" on sperm quality in bulls.

Emerging evidence shows there are "degrees" of heat-sensitivity within species.

As briefly touched on above, recent work from our lab unearthed the idea that even within the same breed of animals (in our case, bulls) there were some animals that displayed remarkable sensitivity to heat whilst others (despite going through exact same hypothermic conditions) were totally unaffected in terms of their sperm quality output. This led to the idea that there are heat-sensitive and heat-tolerant animals. As all enthusiastic scientists do, we felt at this was a novel observation, however, with the advent of time it appears this phenomena has been "found" before but rarely commented upon. For example, in previous studies, six bulls were subject to scrotal insulation "by enclosing the scrotum with a sack constructed of insulated material held in ptlace by Velcroa fasteners and medical tape. Scrotal sacks were fashioned from two layers of waterproofed nylon taffeta filled with a l-cm insulating layer of polyester batting. The layers were machine quilted together and then sewn into a sack. After the first 30 mm of scrotal insulation, allowing for thermal response of the scrotum to the elevated temperature, the sack was readjusted to ensure complete coverage of the scrotum and scrotal neck up to the body wall." (cited from [70]). The bulls had access to the same water and food and were basically subject to the same environmental conditions throughout the work. However, after 9-30 days, two animals showed large increases in abnormal spermatozoa (~65 and 69%), three animals had between 47-51% abnormal sperm and one animal had fewer than 24% abnormal forms [8,70]. As such, there was a large difference in bull thermotolerance, suggesting individual bulls can display degrees of thermosensitivity. Supporting this, 48 hours scrotal insulation of 4 bulls leads to normal forms of one animal dropping to a staggering, 0.5%. Bulls 2 and 3 fared slightly better, dropping to 22% and 29% respectively. However, the 4th bull, despite undergoing the same experimental insult, did not respond to thermal insulation, and maintained 82% normal forms for 3 weeks [71]. Finally, in our own work, we found definitive evidence that some bulls were, and others were not thermosensitive. By taking 20 bulls and purposefully heating them in a shed, we found large changes in semen quality following intervention. Some bulls dropping to below 50% normal forms, whilst others maintained their baseline 80% average throughout 12 weeks of testing (ref).

The idea of thermo-sensitivity is not restricted to bulls, but the evidence points to this being a phenomenon in other animal model systems and we propose all mammals with a scrotum. For example, within Boars used for artificial insemination (AI) it has been noted previously that some of these lines appeared to have a high "heat-tolerance" [72]. Indeed in many water-bath models one finds large difference in the response of animals, despite the fact that they go through the same experimental regime (ref).

Evidence of Genetic Heritability of "Testicular Heat-Sensitivity"

Although scarce, there is some data suggesting heat-sensitivity could be inherited. As outlined above, within Boars used for AI, there is significant variation in sperm production and differences among the genetic lines [72]. Some of these lines appeared to have a high "heat-tolerance", compared to others. For example, in one lineage, high environmental temperatures lead to a 5-7% decrease on sperm output, significantly better the 2 other linages in which the same amount of heat stress causes a 15-20% decease [72]. In addition in Drosophila, the idea of heat-sensitive and heat-tolerant males has also been reported. Heat sensitive *Drosophila* males, that produce no or a low numbers of maggots after a heat event. Remarkably, the "trait" is heritable. Heat-sensitive males produce "heat-sensitive" male offspring [73]. Conversely, heat-tolerant males produce heat-tolerant offspring [73]. Even when fly's with different thermosensitive are used to breed with the same female, the offspring also follow the trait of the male. As such, thermo-sensitivity in *Drosophila* is thought to be passed on from sire to son through the Y-chromosome [73].

Testicular heat Stress and its Relation to Male-Factor Infertility

Although the impact of testicular hyperthermia is often studies in the connect of agriculture, there is little doubt that it also plays a significant role in humans. Male infertility is a medical condition affecting one in 20 men in the western world [74,75]

and accounts solely, or in a contributory way, for ~50% of couples attending assisted conception (AC) [76]. Whilst some infertile men have associated conditions, such as (i) varicoceles [77-79] (27% of cases) or (ii) cryptorchidism [80-83] (6% of cases), others do not and fall into the category of "idiopathic infertility" – or infertility of unknown aetiology (35% of clinical load). In all cases, these men produce an abnormal semen profile. Either their ejaculate contains low sperm counts, low sperm motility, low sperm morphology [84] or (as if often the case) a combination of these phenotypes. In addition, infertile men produce 2-3 times higher levels of DNA damaged spermatozoa compared to their fertile counterparts [85,86]. This contributes to the problem, as men with high levels of sperm DNA damage (defined as 40% of sperm population) are essentially infertile [85-87].

In clinical trials, many dating back to the 1980s, scrotal cooling has been shown to be an effective proven alternative form of assisted conception that improves semen quality and natural pregnancy rates. For example, trials performed in 25 infertile men showed that in 18 men (70%), semen parameters improved with scrotal cooling [88], and 6 (24%) went on to conceive a natural pregnancy during the 14-week scrotal cooling regime, despite the fact they had been "trying" to conceive for 3-8 years previously [89-91]. To put this into perspective, if a couple has not conceived within 2 years of trying, their chance of conception thereafter is less than 2% [92]. A second trial involving 64 men, showed improvements in semen parameters in 66% of cases and a pregnancy rate of 27%[93] within 16 weeks of scrotal cooling. This was statistically significant as the background pregnancy rate (men with poor semen that pulled out of the trial after two weeks) was 5% [93]. However, if we re-analyse the data, and remove men from the analysis who were azoospermic (produce no sperm in their ejaculate) or severely oligozoospermic (<1 million sperm/mL in ejaculate) this increases the pregnancy success rate to 50% [93] against a background rate (men that pulled out of the study) of 10%. Three further studies have looked at pregnancy rates following scrotal cooling ith remarkable outcomes (24% [93], 27% [94] and 14% on a background rate of less than 5% in men with history of fertility of at least 3 years). Moreover, these studies were done over 14, 16 and 8 weeks, respectively. Considering sperm parameters take 4-8 weeks to improve with scrotal cooling, this leaves only 1-2 month window to achieve a pregnancy which is a remarkable success rate. As such, it is clear that in around 40-80% of infertile/subfertile men, scrotal heat stress is a physiological phenomenon that can be overcome.

Concluding remarks

Several sources of environmental stress have been suggested to affect semen quality, yet none of them hold up the rigour of scrotal hypothermia. Whilst we do not yet understand the biochemical mechanisms nor reason why male-precursor germ cells need to remain at or below 34oC, it is clear that this must be the case. Once temperatures exceed 34°C, spermatocytes tend to undergo apoptosis, whilst spermatids continue to develop into misshapen spermatozoa. Yet the environment may not be the end of the story. The emerging role of genetics, and expression levels of different proteins/enzyme will likely explain the concept of "thermosensitivity". The biggest "hint" in terms of causation, is the case of *drosophila*, where heat-sensitive male produce other heat-sensitive males. Further work to understand the mechanism of testicular heat stress, and answer the age-old question of why the scrotum exists will certainly be useful not just from a medical point of view, but also in the agriculture industry to identify males that are heat-resistant.

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