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The seasonal effect of temperature on bull semen quality: The emergence of heat-sensitive and heat-tolerant bulls.

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Abstract

A key challenge for years to come is feeding a rapidly growing human population, whilst lowering the overall costs and impact on the environment. In addition, as global temperatures increase, natural resources must be utilised as a basic means to overcome climate-related issues. To this end, improvements to bull fertility are recognised as a major mechanism that address both the economical and environmental balance. Using semen data from 1271 ejaculates (79 different bull, 11 different breeds) we have investigated the variability of semen quality in cattle living in sub-tropical conditions. Modelling show definitive evidence of seasonal variation, with 90% cryopreservation pass rates winter, dropping to less than 50% in summer. Individual bulls could be classified as either “heat-tolerant” (produce good quality spermatozoa all year regardless of temperature) or “heat-sensitive”. Nominal logistic regression demonstrated when temperatures reach 30.5°C, 40% of heat-sensitive bulls fail a semen analysis 17 days later. At 34°C, the proportion of bulls failing will reach 63%. Ratifying this, the purposeful heating of bulls to 40°C for 12 hours experimental verified the dataset, demonstrating heat-tolerant and different degrees of heat-sensitive bulls exists. Notably, heat-tolerance and heat-sensitivity appear to be genetically inherited. Using historical temperature data, we then modelled how many days/decade bulls would be subject to heat-events. Beginning from 1939-1949, on average, the area in which bulls were kept recorded 19, 7 and 1 day over 38 °C, 39 °C and 40°C. This number steadily increases and of last decade (2010-2019), the numbers of days per decade over 38 °C, 39 °C and 40 °C jumped to a staggering 75, 39 and 15 respectively. These data show the urgent need to identify heat-tolerant bulls as future sires. Such genetic gain would decrease the generational interval, prevent unnecessary culling of cows and increase efficiency of livestock needed for world food programs.

Introduction

A key challenge for years to come is feeding a rapidly growing human population, whilst lowering the overall costs and impact on the environment. Whilst beef is consumed in every country, ruminant livestock produce between 250-500 L of methane per day, and account for 11-17% of methane generated globally ¹. Yet food security is central to the 2030 UN Agenda of Sustainable Development Goals with the aim to end poverty and protect the planet from further devastation ². To achieve these goals, food production must increase to meet demand, however, farming practices are all looking for ways to become more efficient in terms of food costs as well as reduce environmental impact ¹.

To this end, improving cattle fertility is recognised as a major way to overcome this global issue. Improved fertility rates lead to less wastage, improved economic viability and decreased food costs. Whilst improvements in cow fertility have been achieved ³, the fertility of a bull should not be overlooked as one animal will often serve multiple cows, arguably making an individual bull's fertility as, if not more, important. The rare condition of complete sterility can be easily identified in bulls through semen analysis, observation of anatomic abnormalities or by a high rate of service return ⁴. In contrast, sub-fertile bulls are more problematic to be identify. Sub-fertile bulls delay conception, resulting in prolonged calving periods and reduced weaning weights. In addition, the "wastage" caused by subfertile bulls is significant. Non-pregnant cows are often culled needlessly requiring replacement and excess bulls are often put out to paddock in order to compensate.

To determine the fertility potential of a bull, a qualified veterinarian performs a veterinary Bull Breeding Soundness Evaluation (vBBSE). The vBBSE assesses the animal, including measurement of scrotal circumference, sperm motility and sperm morphology. The values obtained are then compared to a "normal" range of parameters and a predictive measure of the *in vivo* fertilisation potential of the bull is given. Typically, the benefit of a vBBSE is a risk assessment to determine the likelihood of a bull being sub-fertile. Although correlations between the vBBSE test and calf output have been reported for 70 years ⁵⁻⁸ it is very clear that the ability of the vBBSE to predict actual fertilisation rates is very inconsistent. For example, collection of semen from 12 Holstein bulls (ranging from 6-11 years old) demonstrated that

total sperm motility had a very poor predictive value to actual conception rates ($r^2=0.34$). When progressive motility was measured, the predictive value increased ($r^2=0.68$)⁹, which could be further improved by combining at least 5 different measurements of motility (lateral head displacement, beat-cross frequency, linearity, path velocity, straight line velocity). However, analysis of data from 117 young artificially inseminated bulls with at least 100 services each showed sperm motility was an extremely poor predictor of fertility with an r^2 value between 0.13-0.24¹⁰. Correlations between bull fertility and sperm morphology¹¹⁻¹³, or cell viability have also been performed and range from 0.06-0.86 and 0.33-0.66 respectively¹¹⁻¹³. Although these correlations are an improvement over motility, they are by no means definitive. In Australia, the “bull power project” was established, consisting of 1000 bulls in the northern herds with the aim to correlate the vBBSE with calf output in higher numbers of animals. However, when “% normal morphology” was correlated to calf output, their conclusion was the same as Johnson¹⁴ in that there was no satisfactory relationship between bull fertility and sperm morphology¹⁵.

One of the issues in trying to correlate sperm parameters to fertility is studies performed in the 1940's have shown that some bulls demonstrate a great variation in sperm quality¹⁶. In 1978, the source of the variation was investigated using 5033 ejaculates from 55 mature Holstein bulls. From this analysis, there was a modest increase (9%) in total sperm from the first ejaculate compared to the second. However, seasonal variation in semen quality was shown, with winter months being the lowest compared to summer ($3.4-3.7 \times 10^9$ vs 4.2×10^9 total sperm). Yet further work demonstrating the effect of season and bull sperm quality has led to largely inconsistent conclusions. For example, whilst some have shown no seasonal effect on sperm motility,¹⁷⁻²¹ others have shown an increase during summer^{22,23} or (in complete contrast) winter²⁴⁻²⁶. Similarly, results have been obtained with total sperm production and sperm morphology with some reports suggesting season has no effect^{17,18 19,27} whilst others show an increase during summer^{18,22,23,28}, or winter^{17,20,21,27}. As such, opinions on whether seasonal alterations are indeed a source of variation in cattle semen quality are still divided. However, it is important to highlight that a closer examination of these studies shows that much of the contradictory results may arise from the fact that these experiments were performed at very different locations. For example, in both Kenya and Thailand in which ambient temperature is consistent throughout the year, little variation in semen quality was observed^{17,21}. However, in USA¹⁹ and Sweden²⁴ where winter temperatures drop below zero, it is little surprise that sperm motility is higher during summer.

In addition to season, semen is also known to change as a result of bull age, nutritional status and breed ^{19,29,30}. Therefore, the objective of this study was to understand what are the major sources of semen variation of bulls maintained in the sub-tropical climate. This knowledge will guide the proposal of practical measures, allowing an easier identification of sub-fertile bulls (e.g., changes to the vBBSE recommendations) and the improve in fertility, which will ultimately result in decreased food costs.

Results

Intra-bull semen quality variation

To understand variation in bull semen quality, we assessed 1271 ejaculates from 79 different bulls (11 different breeds) housed at Rockhampton stud farm, in the state of Queensland, Australia, over a period of 5 years (2014-2018). The raw data is available in Supplementary 1. The climate in this area (23.3786° S, 150.5089° E) is considered sub-tropical, ranging from 16°C in winter to over 30°C in summer. A comprehensive semen analysis was undertaken, including sperm morphology and motility. To determine the variation in semen quality, we plotted the percentage of sperm normal forms for each bull that had 5 or more ejaculates taken annually. Morphology was used as a measure of sperm quality, as Methevon *et al.* ²¹ demonstrated that sperm motility is heavily influenced by the collection/collectors and, therefore potentially unreliable and irreproducible. This resulted in the analysis of 1178 ejaculates from 50 bulls, with an average of 23 ejaculates per bull. The percentage of sperm normal forms as a box and whiskers plot for each bull is given (Fig. 1). As shown, many bulls demonstrated extremely high variation between ejaculates, with several males ranging from <50% normal forms (considered an outright “fail” in terms of cryopreservation potential) to >70% (considered an outright “pass” in terms of cryopreservation potential) of normal sperm morphology. On the contrary, some bulls appeared to produce consistent semen samples across the year.

To determine the amplitude and the proportion of bulls demonstrating variation in the number of normal sperm forms, we measured the difference between the maximum and the minimum values recorded for each animal. From this analysis we found that: 9 (18%) bulls showed less than 20% variation in normal forms; 15 (30%) bulls had between 20-40% variation; 13 (26%) bulls were between 40-60% and for 13 (26%) bulls this number was over 60%. These data have major implications when interpreting semen analysis, since a bull could be classified as either fertile or infertile depending on which ejaculate was considered. This data also sheds

light into why correlations between the vBBSE parameters such as morphology and the bull fertility are so variable.

Seasonal effect on semen quality

Several sources of environmental influence have been suggested to affect bull sperm quality. These include feed availability (i.e., higher conception rates in rainy seasons)³¹, excessive protein intake³², day length³³, thermal heat stress and age^{34,35}. To better understand the dynamics of semen quality variation within our samples, we plotted sample “pass” and “fail” cryopreservation criteria against the month of collection. A raw bull semen sample is classified as “pass” when motility is above 60% and normal forms greater than 70%. When samples were between 30-60% motility and 50-70% normal forms, they were classified as a “compensatory (or qualified) pass”. The compensatory pass relied on there being the ability to have at least 10 million motile normal forms of spermatozoa in each straw to allow for conception. An outright failure was given to any sample with less than 30% progressive motility or 50% normal forms. This allowed each ejaculate to be placed into a binary “pass” or “fail”.

The data for the percentage of total males that “failed” within each month (1271 ejaculates) is shown (Fig. 2A). Clearly, there is a seasonal pattern, with over 90% pass rate in winter (June-August) that fell to 50% or lower in summer (Dec-Feb). Considering that all bulls were greater than 4 years old, housed on the same stud farm and received the same dietary supplement we found no relationship in terms of “pass” or “fail” rates to these parameters. Thus, the data clearly suggested that Temperature/Temperature-Humidity or day length were responsible for the increased failure rates seen during Summer. Therefore, to understand if there was any causal relationship, we correlated either the average monthly temperature (Fig. 2B) or daylight (Fig. 2C) with monthly failure rates. The data showed a correlation with monthly temperature ($r^2=0.55$; and temperature-humidity index – see further modelling below, data not shown) but not with daylight hours ($r^2=0.05$). Combined, these data suggest that temperature was the most likely reason for increased failure rates during the warm/hot months.

Changes in normal sperm forms categorised by breed

The present study investigated 11 different breeds of cattle, and we reasoned that maybe one, or more breed(s) contributed to failure rates more than others. Therefore, we plotted the percentage of normal forms for every ejaculate against the breed (Fig. 3). All breeds showed similar variation with the exception of the Belmont Red, Boran and Wagyu. However, a

relatively small number of bulls from the Belmont Red and Boran breeds were assessed in this study, therefore, it is unclear if they are indeed more resistant to heat. In the case of the Wagyu, it is worth mentioning that only one animal exhibited poor sperm morphology in several ejaculates (Fig. 3 circled) during winter. A close inspection of the records showed that during this time the animal had a fever episode, with body temperature reaching 39.4°C, and that the sperm morphology went back to normal in approximately 70 days.

Some bulls are heat-sensitive, whilst others are heat-tolerant

Analysis of the present data clearly illustrated that some bulls showed marked variation in terms of their semen quality throughout the year (Fig. 1). Meanwhile, others demonstrated much less variation, and were reasonably consistent. To further clarify these differences, we closely analysed the percentage of sperm morphology from two bulls, both of whom had several ejaculates taken throughout the year, including during and after summer (Fig. 4). There was a clear pattern, and evidence of two types of bulls. Prior to the summer season, bull 1 (Fig. 4, red), designated here as “heat-sensitive”, exhibited >70% normal forms of spermatozoa. This value decreases dramatically, reaching its lowest point (10%) mid-January, before undergoing a recovery by April (>70%). In contrast, bull 2 (Fig. 4, green) showed a consistent semen profile throughout the year. The data suggest this bull was more “heat-tolerant”.

To further explore the concept of “heat-tolerant” and “heat-sensitive” bulls, we took 20 Wagyu bulls and subjected them to a single event of controlled heat stress (40°C, 12h). This experiment was done during Winter, at Singleton (New South Wales, Australia, 32.5695° S, 151.1788° E), where the average temperature was 17°C and never exceeded 18°C. Before the heat stress event, baseline semen samples were taken from each animal. After heat stress, semen samples were taken every week for 11 weeks. During the experiment, two bulls were removed from the program due to infection and sickness whilst a 3rd bull was removed as it refused to co-operate with electroejaculation procedure. From the remaining bulls, we were able to reproduce the heat-sensitive and heat-tolerant bull phenomenon. The raw data from this work is given in Supplementary 2, and an example of the data is shown (Fig. 5). For 14 bulls, we found no difference in terms of their baseline samples, which were between 70-90% normal forms. This is consistent with the Wagyu bull characteristics and their heat-tolerance (Fig. 5, yellow, green, blue lines). Within these “heat-tolerant” bulls, there was a variation of 16-22% sperm normal forms. For the other three bulls, two of them showed a decline in sperm quality,

which began 2-3 weeks after the heat event, dropping from a baseline of 85% and 90% normal forms to 55% and 59%, respectively (30-31% variation in normal forms; Fig. 5, grey and orange line). The third bull showed a greater degree of heat-sensitivity. Starting at 77% morphologically normal sperm, the spermogram of this bull illustrated a rapid decrease in normal forms in a short time (2 weeks), reaching around 40% after 4-5 weeks. Sperm morphology remained at this level (37% variation in normal form) for four weeks, before recovery. These data show that under experimental condition, the phenomenon of heat-sensitive and heat-tolerant animals can be reproduced. Further, it appears that there are degrees of heat-sensitivity.

Environmental heat stress leads to poor sperm quality 17 days later

Similar to previous reports, we noted that sperm quality does not begin to deteriorate until 2-3 weeks after the heat stress event of the bulls³⁶. Based on the timing of spermatogenesis, this is consistent with reports that meiotic cells are more susceptible to heat stress following a heating event, with poor quality spermatozoa appearing in the ejaculate around 2-3 weeks later. To better understand the relationship between a “heat-event” and the production of poor quality spermatozoa, we modelled both maximum temperature and maximum temperature humidity index (THI) and their relationship to the proportion of morphologically normal spermatozoa. The THI is an index representing the effect of humidity on the heat stress of an animal. THI was obtained using the following formula:

$$THI = 0.8 * T_{max} + \frac{(humidity * (T_{max} - 14.4))}{100} + 46.4$$

where T = maximum temperature (°F), and H = relative humidity.

Initially we assessed both maximum temp and THI on the day semen was collected, and correlated this with percentage sperm normal forms. Modest negative correlations were found indicating that as temperature increased sperm quality decreased. We reasoned that if meiotic cells (MII spermatocytes/spermatids) are heat-sensitive, then temperatures leading up to the day of semen collection should improve the correlation. This was therefore investigated by determining the correlations between both T_{max} and THI every day prior to the semen sample collection date for up to 40 days (i.e. if semen collection day is designated Day 0, we looked at temperatures on day -1 through to day -40). The correlations between both T_{max} (Fig. 6A) and THI (Fig. 6B) to percentage sperm normal forms for each day prior to collection

is shown. In this case, the highest correlation was found 17 days before semen collection (Fig. 6A/B, panel 1, black arrows).

In addition to looking at a single day of heat (Fig. 6, panel 1), we explored the possibility that a “heat-event” of 2 or more days is required in order to trigger poor sperm production in bulls. Therefore, to model this, we averaged the temperature (Fig. 6A) or THI (Fig. 6B) across either 2, 3, 4 or 5 consecutive days (panels 2-5) to see if correlations improved. The correlation patterns achieved (panels 2-5) were similar in these plots to the individual day plots (panel 1) with only modest improvements in the maximum (negative) correlation achieved. In almost all the correlations patterns in Fig. 6 the maximum correlation was achieved at 17 days. From a practical perspective, this demonstrates that the detrimental effect of temperature on the quality of spermatozoa produced on the day that semen was collected, was a function of the heat event that occurred 17 days prior. Furthermore, it is evident that even one day of heat stress can lead to poor quality spermatozoa.

Understanding the temperatures at which heat-sensitive bulls fail

To determine the T_{max} at which bulls in the paddock begin to produce poor quality spermatozoa, we modelled data using both parameters measured at 17 days prior to the heat event, and plotted samples from 12 heat-sensitive bulls (6 Brahmans, 4 Drought Masters and 2 Santa Gertrudis). The relationship between sperm morphology and T_{max} 17 days prior to heat even was plotted, with a spline smoothing curve to show the mean quality as a function of T_{max} (Fig. 7a). As the temperature increase, so the quality of sperm morphology decrease as expected. In order to gain further clarity, we next fitted a nominal logistic regression analysis to model the proportion of spermatozoa that would either pass, Q-pass or fail sperm cryopreservation criteria as a function of T_{max} 17 days prior. T_{max} effect was highly significant for both outcome categories, with both $p < 0.001$. The predicted proportions from the model for each pass condition at varying T_{max} were plotted (Fig. 7B). At lower temperatures ($<22^{\circ}\text{C}$), the proportion of samples that would be expected to pass (0.90, solid line) or qualified-pass (Q-pass) (0.08, dotted line) summates to 0.98. This is similar to the actual number of samples that pass during winter (Fig. 2). However, as temperatures rise, the number of samples designated as “pass” begins to drop, while the number of qualified-pass samples increase up to 30.5°C . These data show that a “q-pass” is likely reflecting the beginnings of the effect of heat stress. At this point, the proportion of samples with a q-pass

has reached 0.38, whilst the proportion of samples with a direct “pass” has dropped to 0.22, giving an overall 0.6 probable pass rate. At 34°C, the proportion with an overall pass (pass 0.07 + q-pass 0.30) drops to 0.37 and continues to drop as temperatures rise above 34°C. From a practical perspective, this data show that for heat-sensitive bulls, with temperature histories 17 days earlier of around 31°C, 40% of the sperm samples collected will fail a spermiogram assessment and once the temperature exceeds 34°C the failure rate will exceed 63%. As such, these data suggest that within heat-sensitive bulls, there are clearly some bulls that are more sensitive than others.

Climate change modelling shows bulls are subjected to more heat events per decade

To understand the impact of global warming, we downloaded historical temperature data from the Bureau of Meteorology using data from the Rockhampton site (bom.gov.au), which is also available in Supplementary 3. We then counted the number of days/decade the maximum temperature exceed 32°C-45°C respectively. These data are shown in Table 1. To give an example of “extreme” climate that would likely impact all heat-sensitive bulls, we plotted the number days/decade which were higher than 38°C, 39°C and 40°C (Fig. 8) from the area in which the bulls were housed. As shown, the number of days per decade over these extreme temperatures jumps from 19, 7 and 1 (1939-1949; 38°C, 39°C and 40°C respectively) to a staggering 75, 39 and 15 in the last decade. These data show the urgent need to identify heat-tolerant bulls as future sires. Such genetic gain would decrease the generational interval, prevent unnecessary culling of cows and increase efficiency of livestock needed for world food programs.

Discussion

In 1938, the first report of seasonal effects on breeding efficiency in dairy cattle was shown when the University of Nebraska dairy herd records demonstrated more services were required for conception during summer (May to October) from 1896 to 1934³⁷. This same trend was also seen in records obtained from Purdue³⁸ and Louisiana state university³⁹. However, these studies were contradicted by reports from Cornell University, whose records showed winter was the poorest season for breeding dairy cattle in Canada and New York State^{40,41}. At this stage, the underlying presumption was that the cow fertility was more of an issue than the bull fertility, and it was the cow that was somehow affected by heat. Indeed, in dairy cattle it is now well established that increased THI has a devastating effect on cow production and fertility, particularly, on early embryo development⁴². It was not until 1941, when Anderson analysed

sperm volume and motility, and showed during summer in Kenya ⁴¹ that these two parameters were poor. Noteworthy, sperm volume and motility are likely the poorest “predictors” of bull fertility known today. Combined with experimental data on the heating of dairy bulls inside chambers (32.2°C), there was evidence to show that a warm environment can lead to a decrease in sperm morphology and motility ⁴³.

Despite this data, controversy surrounding “season” and bull fertility continues to this day, with some reports showing no seasonal effect ¹⁷⁻²¹, whilst others have improved semen quality in winter ^{20,21} or summer ^{22,28}. However, inspection of that data shows the seasonal effect of heat stress can largely be explained by climate. Better sperm parameters in summer typically occurs where places experience sub zero temperatures in winter. Studies where season does not affect bull sperm quality are often in areas where temperature and humidity do not change substantially.

Our current work, which investigates predominately *Bos indicus* breeds of cattle used for meat purposes, shows season definitely has an effect on bull semen quality. During winter (25°C), samples had over 90% pass rate, whereas in summer (32°C) this dropped below 50%. However, not every animal responds to temperature to the same degree even those subjected to the exact same environmental conditions. For this reason, we suggest there are heat-sensitive and heat-tolerant bulls. In addition, to this, there are “degrees of heat-sensitivity”. From our data, it appears that roughly half of the bulls analysed had a higher degree of heat-sensitivity, with poor sperm production occurring in 63% of heat-sensitive bulls once ambient temperatures exceeded 34°C. Modelling of the temperature before the day of semen collections showed that poor quality spermatozoa do not appear until 17 days after the heat stress event. This is consistent with our understanding of the heat-sensitive nature of meiotic (secondary spermatocytes) cells in the testis. However, for roughly 40% of the animals in this study, they seemed to be totally unaffected by temperature, despite experiencing the same conditions. These animals produced normal forms of spermatozoa throughout the entire year. In retrospect, our observation of heat-sensitive and heat-tolerant bulls may explain some of the previous data in experimental models. For example, in previous studies of six bulls that were subject to scrotal insulation, two showed large increases in abnormal spermatozoa (~60%), whereas others had fewer than 23% abnormal forms ^{44,45}. Furthermore, after 48 hours scrotal insulation of 4 bulls, normal forms of spermatozoa had dropped to 0.5% in the first bull and 22% and 29% in bulls 2 and 3, 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 ⁴⁶. Thus, although not formally recognised nor commented on, it appears that heat-

sensitive and heat-tolerant bulls have been previously demonstrated using other experimental models.

From the data we analysed, the Wagyu breed stood out, in terms of heat-tolerance. Using temperatures of 40°C (where according to our model, most bulls should fail), we managed to show remarkable heat-resistance in the Wagyu. In fact, several weeks post heat stress insult, only 3 of the 17 bulls started to show the typical signs of testicular heat stress, including poor sperm quality. One bull demonstrated a major change in normal forms, dropping from 70% to 40% over a period of five weeks. The other two bulls showed an intermediate drop from a baseline of 80% to 60% morphologically normal spermatozoa. Yet why is the Wagyu so tolerant? the answer may be genetic. Lineage tracing of the three animals that produced poor quality spermatozoa after heating showed they were half-brothers, same sire and different dams. Yet all the other animals were sired by a different bull. These data point to the possibility that heat-sensitivity could be inherited. This appears consistent in other model systems. For example, heat-sensitive *Drosophila* males (i.e., produce no or low numbers of maggots after a heat event) sire other males that are also heat-sensitive⁴⁷. Conversely, males that do produce maggots following the same heat event sire “heat-tolerant” male offspring⁴⁷. In both cases, the female fly appears to have no control on whether the offspring are heat-sensitive or not. As such, thermo-sensitivity in *Drosophila* is thought to be passed on from sire to son through the Y-chromosome⁴⁷.

The changing variation in semen quality, seen in roughly half of animals investigated here, sheds light into the predictive nature of a semen test for bull fertility and the future of farming practice. The ability of an animal to produce over 70% normal forms in one ejaculate, yet drop below 10% during summer, explains why the vBBSE is a poor predictor of fertility. Additionally, identification of heat-sensitive bulls and removal from the herd/breeding pool would not only lift fertility rates but prevent the unnecessary culling of “empty” (non-pregnant) cows and overuse of bulls. Given the staggering rise in the number of days sub-tropical conditions are experiencing (Table 1, Fig. 8) it is no wonder that these areas have reported historical lows in terms of fertility rates⁴⁸. Identification of heat-tolerant bulls would add much genetic gain not only reducing the amount of animals needless used and culled, but as a consequence, reduce global methane emissions and lower food costs

In summary, our data show that bulls respond to heat stress in a different manner, with some bulls being more prone than others. Intriguingly, the *Bos taurus* breed Wagyu shows there is genetic gain to be had that can be achieved as this breed in general show a high resistance to

heat stress. Finally, the future of the vBBSE and its correlation to fertility needs to be properly addressed, accounting for seasonal changes and individual responses to these changes. By taking a summer sample at least 17 days after a maximum temperature of 36°C, over 80% of heat-sensitive bulls should be identified allowing better management and fertility rates in cattle.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise stated at the highest research grade. Other chemicals purchased were Percoll (GE Healthcare, Castle Hill, NSW, Australia); HEPES solution (Gibco, Invitrogen Australia, Melbourne, VIC, Australia); 10 × Hams F10 (MP Biomedical, Seven Hills, NSW, Australia); D-glucose (Thermo Fisher Ajax Finechem, Taren Point, NSW, Australia); methanol (Fronine, Riverstone, NSW, Australia); and Spurr's embedding kit, paraformaldehyde and glutaraldehyde (ProSciTech, Thuringowa Central, QLD, Australia).

Preparation of Bull spermatozoa and ethics

Institutional and State Government ethical approval was obtained for the use of bulls involved in this research programme (A-2020-008). All bulls were allowed to feed *ad libitum* in a paddock, and their diet was supplemented with a mixed ration [68.4% (wt/wt) dry matter, 7.2% (wt/wt) protein, 36.2% (wt/wt) neutral detergent fibre, 20.0% (wt/wt) acid detergent fibre, 1.45 net energy Mcal/kg, and 3.5 g minerals/kg (NaCl, Ca, and P) on a dry matter basis]. The semen samples were collected by electroejaculation no more than twice per week. Samples were collected into a warmed (38°C) sterile 10 mL centrifuge tube and immediately placed into an incubator set to 38°C. As the bulls were being used for stud purposes, often a second sample (recorded in supplementary 2) was taken. An aliquot of cells was taken and viewed under DIC microscope to assess sperm motility. A further 10 µL of sample was fixed in 1.0 mL 10% TBS for sperm morphology analysis.

Climate conditions

The climate of the region where the study was conducted is considered sub-tropical. The climate data for each day from 01 Feb 2014 to 12 Aug 2019 was accessed from the Bureau of Meteorology and is available in Supplementary 2. Monthly average temperatures exceed 30°C

from October to March, then dropped to 23°C in July. The relative humidity all year around generally sat between 80 to 100%, with very few days dropping below this value.

Analysis of semen samples

Animals were left to acclimatise for a month at Rockhampton. The bull was restrained inside a cattle crush and semen was collected by electroejaculation into a warmed 35°C 15 mL plastic vial. Once collected, the sample was immediately placed into a 35°C water bath before further assessment. Sperm motility was subjectively assessed by extending the semen approximately 1:1 with PBS and then scored at 100-400X magnification.

For morphology, 50 µL of raw semen is added to 1-1.5mL buffered formal saline (BFS). An aliquot was then viewed via DIC at 1500X magnification and 100 cells were assessed for morphological characteristics. The sperm morphology traits were individually classified into eight different categories. Bulls that presented less than 70% of normal sperm forms, or at least one value below the threshold established for each morphological category, were considered “fail” for the morphology test. Abnormalities not to exceed: proximal cytoplasmic droplets (20%), vacuoles/teratoid (20%), knobbed acrosomes (30%), pyriform heads (20%), midpiece abnormalities (30%), swollen acrosomes (30%) and loose tails and heads (30%).

Procedure for heat stress of bulls

A pre-existing shed was modified by fabricating an internal insulated wooden structure within the shed. The heating unit was built to 20 m x 3 m x 3m using plywood. A back entrance gate was used to move the bulls in and out from the heating unit. After the plywood framework was built, the sides and roof were then insulated using R3.5 grade wool. The insulation was then covered with a black plastic wrap to allow better control over the air circulation. Two pedestal fans were installed on the roof heating and set to 2100 RPM in order to allow for air circulation within the heating barn. For added insulation, and to maintain 40°C, the ground was covered with a thick 300 mm layer of straw. Eight electric thermometers were placed in even distribution around the barn. Two were placed close to the position of the testis of an average size bull in a standing position. The remaining six were placed in 500 mm increments above or below the latter, allowing the measure of a temperature gradient from the testis to the head of bulls. The temperature measured at the testis site was used as a guide to maintain the

environmental temperature at 40°C. It should be noted that despite fans circulating the air above, a 2°C temperature gradient from head to testis (42°C-40°C) was unavoidable.

In order to heat the room, a 21 kW electric blower heater, capable of airspeeds up to 230 L/sec, was used (Hired from Activeair.com.au). The unit was placed at one end of the shed and ducts were used to direct the air evenly around the heating barn. We conducted a series of tests over the course of three days to determine the appropriate settings and to calibrate the heating unit, thus, ensuring the barn could be heated and maintained at a constant temperature of 40°C. Temperature probes were constantly monitored throughout the 12 h experiment, either directly or via a Bluetooth connection. To regulate the temperature, we found it necessary to have an opening (0.5 m diameter) near one of the duct entrances to the heating barn. As the daytime temperature rose, more air from the outside was allowed in, which maintained a constant 40°C temperature within the building. Throughout the 12 h experiment, fluctuations of around 1-1.5°C occurred. However, by regulating the heating unit air flow and/or by changing the opening diameter, to allow outside air to flow in, these temperature fluctuations never exceeded 3 minutes.

On the day of heating, the barn was heated for 2 h to equilibrate the room to 40°C. The animals were housed inside the shed, but outside of the heating barn, which was a separate room within the shed. After 2 hours, the bulls were introduced into the heating room. Within 10 min, the heating barn had returned to a temperature of 40°C at testicular height, and the 12 h heating period commenced. Throughout the course of the work, the animals had access to water and straw *ad-libitum*. After 12 h of heating at 40°C, the heating unit was turned off and the animals stayed in the heating barn for one hour to prevent cold shock. After this, they were released from the heating unit, but kept under the shed, with food and water *ad-libitum*. The next morning, the animals were released to their paddock.

Statistical analysis

Bulls were classified into two groups, the first group being heat-insensitive if the percentage of a bull's sperm samples that met or exceeded the normal sperm forms pass test (70% or more normal forms) was better than 80%. If not, then a bull was classified as heat-sensitive and only these bulls were used for further analysis. Pearson correlation coefficients were determined between sperm quality % and Tmax or THI for the day the sample was taken (lag 0) and up to lag 40, i.e. up to 40 days prior to the day the sample was taken. The correlations were also

determined with averages of temperatures or THI over a number of days. For example when 2 days were grouped the Tmax or THI values at lag 0 and lag 1 were averaged and correlated with sperm quality % on the day of the sample. The next lag was the average of lag 1 and lag 2 and so on until up to 40 days of history were used. The resultant set of correlation coefficients were plotted on the y axis against the day of the first lag in the group on the x axis. Groupings for the averages were 2, 3, 4 and 5 days. The lag with the largest (negative) correlation was chosen as indicating the point in time when the impact of Tmax or THI on sperm quality most likely took place.

An alternative approach was carried out on a subset of 12 bulls in the heat-sensitive group where each had at least a moderate amount of data (8-44 sperm samples and appeared to display a clear heat-sensitive effect. The analysis was based on converting sperm quality % into 3 categories, passed (sperm quality 70% or better), a qualified pass (50% or better if pass had failed) or failed (less than 50%) as these are criteria for spermogram assessment for cryopreservation. A nominal logistic regression model was fitted to model the proportion of pass and qualified pass as a function of Tmax 17 days prior, the failed category being the reference condition. The predictions from the model were used to prepare a plot of proportion of sperm samples meeting the pass or qualified pass conditions as a function of Tmax 17 days prior.

Figure Legends

Figure 1. Changes in sperm normal forms.

Semen samples were taken from bulls via electroejaculation and the percentage sperm normal forms were counted. The data show a box and whiskers plot consisting of 50 bulls, each of which had at least 5 different ejaculates across a minimum one month. Each box and whiskers plot represents an individual Bull showing the median, upper and lower quartile range. Outliers are represented by individual dots.

Figure 2. Seasonal variation in the semen quality of 1271 bull semen ejaculates

Semen samples were taken from bulls via electroejaculation and a full semen analysis was undertaken. Each sample was then classified as a pass or fail as described in Materials and

Methods. **A)** The percentage failure rate for each month is shown for all bulls. The number above each column indicate how many semen ejaculates were processed that month. **B).** Scatter plot showing the average monthly temperature of Rockhampton and the percentage of samples that fail/month. Line of best fit indicates and $r^2=0.55$. **C)** Scatter plot showing the average daily sunlight in Rockhampton and the percentage of samples that fail/month. Line of best fit indicates and $r^2=0.04$.

Figure 3. Variation in Semen quality as judged by Bull breed.

Semen sample was collected and analysed for sperm morphology. The animals were then separated according to breed and the percentage normal forms for each ejaculate are shown.

Figure 4. Identification of Heat-Sensitive and Heat-Tolerant bulls

The percentage normal sperm morphology from two bulls, both Droughtmasters, which had several ejaculates taken over the course of the year were plotted against the month in which the semen sample was taken. The first bull (red) is an example of a heat-sensitive bull. The second bull (Green) an example of heat-tolerant response.

Figure 5. Heating of Wagyu bulls to identify heat-sensitive and heat-tolerant effect

Twenty Wagyu bulls all 3 years of age and over were heated to 40°C for 12 h in an insulated barn. Before heating, baseline samples were taken (week 1). After heating, electroejaculation was used to collect semen every week for 11 weeks. For every sample, sperm morphology was counted by a qualified theriogenologist. The data show the percentage normal morphology for 5 bulls. The light blue line indicates a heat-sensitive bulls, whose morphology was affected by heat, then returned back to baseline. The orange and grey line represent two related bulls (same father) who also produced less than 70% normal forms. The yellow, green and dark blue lines represent three heat-tolerant bulls, whose semen profile did not drop below the 70% normal spermatozoa threshold.

Figure 6. Bull semen quality (as percentage sperm normal forms) is related to the temperature that occurred 17-19 days ago.

Correlation between sperm quality and (a) maximum Temperature (Tmax) (b) Temperature Humidity Index (THI). The Y axis is the Pearson correlation coefficient and X axis is the lag, i.e. the number of days before the day the sperm sample was taken, at which Tmax or THI was taken to correlate with sperm quality. The panels represent the number of days that were

averaged to form Tmax or THI. The first panel at left is for correlations with Tmax or THI for a single day. The remaining 4 panels contain the correlations between sperm quality and the average of 2, 3, 4 and 5 days of Tmax or THI. The smoother appearance of the correlation pattern in these panels is a result of the averaging of Tmax or THI values over multiple days. The overriding pattern is the correlation between sperm quality and Tmax or THI peaks at about 17-19 days prior to the sperm sample being taken and is substantially greater, ranging from about $r = -.42$ to $-.45$, than it is on the same day (lag 0), about $r = -0.29$, or for longer periods.

Figure 7. Sperm quality as a function of temperature 17 days prior.

Sperm quality was evaluated for a subset of 12 heat-sensitive bulls in two ways, (a) quality as % for each sample taken (b) fitted lines from a logistic regression model estimating the proportion passing for two categories of sperm quality, a pass (quality 70% or better) and a qualified pass (quality 50% or better if pass failed).

Figure 8. Sperm quality as a function of temperature 17 days prior.

Climate change in Rockhampton from 1939-2021

The historical record maximum temperature from Rockhampton weather station (23.38oS, 150.51oE), was downloaded which included data from 1939-2021 from bom.gov.au. The number of days per decade over 38°C (blue), 39°C (orange) and 40°C (grey) are shown.

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Figure 1

Figure 2

A

B

C

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

A

B

Figure 8

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