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Research Article

Temperature-Dependent Thermotaxis and Its Impact on Motility, Viability, and Concentration in Canine (American Bully) Sperm

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ABSTRACT
Sperm transfer through the reproductive tract is crucial for successful fertilization in most of mammals. After ejaculation, spermatozoa need to go through a complex physiological barrier and environments in the reproductive tract. Also, sperm must respond to many environmental conditions, including temperature. The present study aims to investigate the thermotactic response of sperms collected from American Bully dogs and its impact on motility, viability, membrane integrity, and concentration at different temperatures. Evaluated semen samples, collected from mature dogs incubated at 34°C, 38°C, and 40°C for 60 minutes in a custom-designed thermotaxis model. Some sperm parameters were assessed using computer-assisted sperm analysis (iSperm CASA), Nigrosin-Eosin staining, and the Hypo-Osmotic Swelling Test (HOST).Results showed that sperm motility and concentration were increased at 34°C and 38°C, while exposure to 40°C resulted in a significant decline in motility, progressive motility, and concentration. Slightly higher curvilinear velocity (VCL) and straight-line velocity (VSL) at 38°C suggests optimal motility at this temperature. Membrane integrity (HOST-positive sperm) was significantly compromised at 40°C, with a corresponding decrease in viability, as confirmed by Nigrosin-Eosin staining. Sperm direction towards to different temperature for the sample was also affected, with the highest sperm concentration observed at 34°C, followed by 38°C, and the lowest at 40°C, indicating that higher temperatures (likely due to increased oxidative stress and metabolic exhaustion) are not favourable for canine sperm.As conclusion, the results showed that canine spermatozoa exhibit temperature-dependent survival patterns, with an optimal range between 34°C and 38°C. The thermotaxis for spermatozoa may play a role in natural sperm selection, influencing fertilization efficiency. Future studies should be set up to investigate the molecular mechanisms underlying thermotaxis and its potential use in sperm selection for improved fertility outcomes.
Keywords: Thermotaxis, canine sperm, kinetics, motility and viability.

Sıcaklığa Bağlı Termotaksis ve Bunun Köpek (American Bully) Spermlerinde Motilite, Canlılık ve Konsantrasyon Üzerine Etkisi

ÖZ
Memelilerin çoğunda başarılı döllenme için sperm taşınımı, üreme kanalında kritik bir süreçtir. Ejakülasyondan sonra spermatozoonlar, üreme kanalındaki karmaşık fizyolojik bariyerleri ve farklı çevresel koşulları aşmak zorundadır. Ayrıca, spermatozoonların sıcaklık da dahil olmak üzere birçok çevresel faktöre yanıt vermesi gerekmektedir. Bu çalışma, American Bully ırkı köpeklerden toplanan spermatozoonların termotaktik tepkisini incelemeyi ve farklı sıcaklıklarda (32°C, 38°C ve 40°C) inkübasyonun motilite, canlılık, membran bütünlüğü ve konsantrasyon üzerindeki etkisini değerlendirmeyi amaçlamaktadır. Değerlendirilen semen örnekleri, olgun erkek köpeklerden toplanmış ve özel olarak tasarlanmış bir termotaksis modeli içinde 60 dakika boyunca inkübe edilmiştir. Çeşitli sperm parametreleri, bilgisayar destekli sperm analiz sistemi (iSperm CASA), Nigrosin-Eosin boyama ve Hipo-Ozmotik Şişme Testi (HOST) kullanılarak değerlendirilmiştir. Sonuçlar, 32°C ve 38°C sıcaklıklarında sperm motilitesi ve konsantrasyonunda artış olduğunu, ancak 40°C'ye maruz kalmanın motilite, progresif motilite ve konsantrasyonda önemli bir düşüşe yol açtığını göstermiştir. 38°C'de gözlemlenen daha yüksek eğrisel hız (VCL) ve düz çizgi hızı (VSL), bu sıcaklığın optimal motiliteyi desteklediğini göstermektedir. Membran bütünlüğü (HOST-pozitif sperm oranı), 40°C'de önemli ölçüde bozulmuş ve bu durum Nigrosin-Eosin boyaması ile doğrulanan canlılık azalması ile paralellik göstermiştir. Örneklerde sperm yönelimi de sıcaklığa bağlı olarak değişmiş, en yüksek sperm konsantrasyonu 32°C'de, ardından 38°C'de gözlenirken, en düşük konsantrasyon 40°C'de tespit edilmiştir. Bu

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durum, yüksek sıcaklıkların artan oksidatif stres ve metabolik tükenme nedeniyle köpek spermatozoonları için elverişli olmadığını düşündürmektedir. Sonuç olarak, bu çalışma köpek spermatozoonlarının sıcaklığa bağlı olarak hayatta kalma modelleri sergilediğini ve optimal sıcaklık aralığının 32°C ile 38°C arasında olduğunu göstermektedir. Spermatozoonların termotaksisi, doğal sperm seçiliminde önemli bir rol oynayabilir ve döllenme başarısını etkileyebilir. Gelecekteki çalışmalar, termotaksisin moleküler mekanizmalarını araştırarak, bu olgunun fertiliteyi artırmaya yönelik sperm seçiminde nasıl kullanılabileceğini belirlemeye odaklanmalıdır.
Anahtar kelimeler: Termotaxi, köpek sperm, sperm kinetik

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Introduction

Sperm transportation from through the reproductive tracts are important in mammalian reproduction. After ejaculation, spermatozoa need to navigate through various anatomical parts (including the cervix, uterus, and oviduct) to reach the fertilization site in most of mammalian species. This journey also involves very complex physiological interactions, where sperm must pass various biological barriers and respond to environmental conditions.

Recent research in this field have demonstrated that thermotaxis and chemotaxis are two mechanisms, playing critical roles in sperm guidance, in canines. The ability of sperm to be able to detect how to swim toward different temperatures (thermotaxis) or follow specific chemicals (chemotaxis) has been recent topics studied in many species.

Studies have shown that thermotaxis is one of the important navigation ways in canine spermatozoa, like other mammalian species. Khodamoradi et al. (2021) reported that the temperature response of canine sperm has been observed to have directed motility toward warmer temperature zones, suggesting that thermotaxis may have a critical mechanism for sperm selection and fertilization efficiency. Kang et al. (2019) further investigated microfluidic approaches to assess sperm motility and responsiveness to temperature gradients, confirming that canine spermatozoa adjust their swimming direction in response to minute temperature fluctuations.

The role of chemotaxis in sperm movement has been demonstrated in various mammalian species, including dogs, where sperm respond to chemical gradients. Rickard et al. (2022) reported that canine spermatozoa increased motility when exposed to follicular fluid. This result supports that chemical factors play a role in guiding sperm toward the oocyte. Suarez and Pacey (2006) also reported that that canine sperm may respond to molecular signs that occurs during capacitation and fertilization

Ali et al. (2021) found that odorant and taste receptors in canine sperm are directly involved in chemotaxis, suggesting a multi-sensory guidance system suggesting that the combined influence of thermotaxis and chemotaxis on sperm movement toward the oocyte. Additionally, Van de Hoek et al. (2022) showed that sperms require energy for motility and suggested that metabolic adaptation in canine sperm might influence their ability to respond to environmental signals.

Despite the growing research on thermotaxis in canine spermatozoa, many aspects are under investigation. The precise mechanisms regulating thermotaxis in canine sperm have yet to be fully understood. Also, the influence of sperm metabolic adaptations on their ability to detect and respond to temperature gradients remains unclear. Further investigation is also needed to determine whether these mechanisms can be manipulated to enhance assisted reproductive technologies (ART), artificial insemination in canines, as well as other mammals.

This study aims to investigate the impact of thermotaxis responses in canine spermatozoa, with a particular focus on their role in sperm selection at varying temperatures to determine how temperature gradients influence sperm motility, directionality, and viability.

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Materials and Method

Semen Handling

This study did not involve any chemical or physical intervention on live animals. Semen samples were obtained from reproductively mature American Bully dogs kept at a private breeding facility. Collection was performed using a non-invasive manual stimulation method, ensuring minimal stress to the animals. Immediately after collection, the semen was transferred into pre-warmed 50 mL sterile tubes to maintain viability.

The collected semen was transferred to the laboratory in a thermos at 38°C for further processes. At the arrival, A macroscopic examination was carried out to evaluate colour, volume, and viscosity before further analysis.

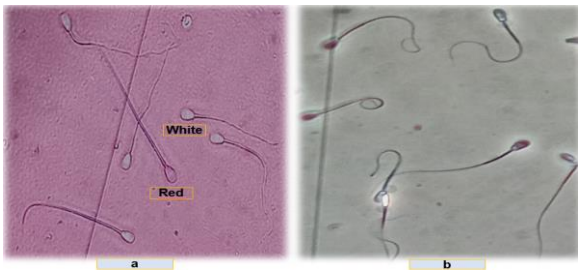
Sperm Analysis Using Computer-Assisted Sperm Analysis (CASA)

Each semen sample was assessed using the Computer-Assisted Sperm Analysis (CASA) system (iSperm, Aidmics Biotechnology), an automated system ensuring objective and high-precision motility and concentration measurements.

Sperm Viability and Membrane Integrity Assessment

Sperm viability was determined using the Nigrosin-Eosin staining technique. A 10 µL sperm sample from each group was mixed with 10 µL of Eosin-and 20 µL Nigrosin stain on a glass slide. After 30 seconds of incubation at room temperature, the mixture was spread into a thin smear by another glass slide and allowed to air dry. Microscopic evaluation was performed under x400 magnification by Leica DMR Phase contrast microscope, by counting at least 100 spermatozoa per slide. Spermatozoa with stained (pink/red) heads were classified as dead, while those with unstained (white) heads were classified as alive. The percentage of dead spermatozoa was recorded for statistical analysis.

Figure 1. a) Nigrosin-Eosin staining and b) Hypo-Osmotic Swelling Test



Membrane integrity assessment was carried out by using the Hypo-Osmotic Swelling Test (HOST). A 100 µL semen sample was mixed with 900 µL of a pre-prepared solution (100 mOsmol fructose, as described by Söderquist et al., 1997) and incubated at +37°C for 30 minutes. Subsequently, a 10 µL aliquot of the mixture was placed on a slide and examined under a Leica DMR phase-contrast microscope. A total of 100 spermatozoa were counted, and the proportion of spermatozoa exhibiting tail curling and swelling was recorded as an indicator of membrane integrity, expressed as a percentage.

Experimental Setup and Thermotaxis Model Design

A custom thermotaxis model was specifically developed for this experiment to assess sperm motility in response to thermal gradients. Detailed specifications of this design and implementation are given below.

Two modified 50 mL Falcon tubes (Figure 2) were utilized for this study. Each tube was divided into two equal compartments by inserting a thin (2 mm) Styrofoam barrier, designed to prevent heat conduction (shown in blue in Figure 2). This barrier also restricted sperm movement between the compartments, which was filled with 20 mL of Sperm-TL solution.

Two Falcon tubes were placed inside an incubator, with one tube assigned for thermotaxis analysis and the other designated as a control. In the thermotaxis tube, the two opposing compartments were subjected to differential thermal conditions using adjustable heating plates. One compartment was maintained the temperature at 34°C, while the

opposite was set to 40°C, thereby establishing a temperature gradient for the experiment.

Temperature regulation was achieved through continuous measure of temperature by sensors positioned within each compartment of the Falcon tube. The heating plates were adjusted accordingly to maintain the target temperatures within a ±1°C range, ensuring stable thermal conditions throughout the experiment.

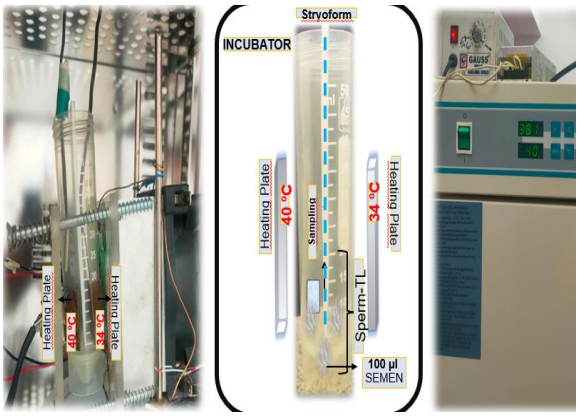


Figure 2. Thermotaxis Model Design

Semen Sample Preparation and Incubation Conditions

Freshly collected semen samples, exhibiting a minimum 75% viability, were used for the study. A 100 µL aliquot from each sample was carefully placed by using 200 µL Eppendorf pipet into the conical bottom of both the control and thermotaxis falcon tubes, which had been filled with 20 mL of Sperm-TL solution and incubated at least 2 hours early. This process was conducted with precision to minimize sample dispersion and disruption.

The sperm-containing Falcon tubes were subsequently incubated for 60 minutes under controlled atmospheric conditions (5% CO_2 concentration) and maximum humidity. The control tube was maintained within the same incubator at 38°C (Table 1), while the thermotaxis tube was exposed to the 34°C–40°C thermal gradient.

To evaluate thermotactic behaviour, the movement of live and motile spermatozoa towards different temperature zones was assessed using the swim-up technique.

Sample Collection and Analysis

Following the 60-minute incubation period, 1000 µL of sperm suspension was collected from each of the two equally sized compartments (Figure 2) for concentration and morphological analysis. These samples were subsequently processed to assess thermotactic response in relation to the established temperature gradient.

Table 1. Experimental Temperature.

Group	Incubator (ºC)	Falcon (ºC)
Control	38	38 - 38
Thermotaxis	38	34 - 40

Statistical analysis

In the present experiment, three temperature gradients (34°C, 38°C and 40°C) were used in a randomised design for sperm thermotaxis. Statistical analyses were performed using one-way ANOVA to assess the effect of temperature on canine sperm kinetic parameters. Tukey's Honestly Significant Difference (HSD) test was used to determine pairwise differences between temperature groups The data obtained from the experiment were analyzed using Minitab 19 package program.

Results and Discussion

Sperm Concentration

The sperm concentration data, presented in Graph 1, show that temperature influences sperm retention in the sample after 60 minutes. The highest sperm concentration was observed at 34°C, followed by 38°C, while the lowest concentration was recorded at 40°C. This suggests that higher temperatures may lead to increased sperm loss, potentially due to cell damage or premature activation of metabolic processes leading to reduced survival. The decline in sperm concentration at 40°C aligns with the observed reductions in motility, viability, and membrane integrity, reinforcing the negative impact of excessive heat exposure on sperm preservation.

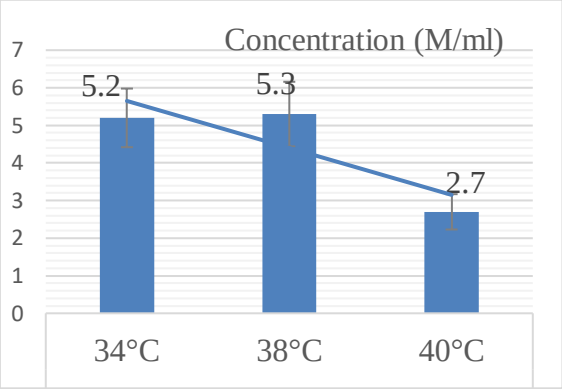


Figure 3. Temperature impact on canine sperm concentration

Sperm Motility and Kinetic Parameters

The motility analysis revealed that incubation temperature significantly influenced sperm movement characteristics. The highest total motility was observed at 34°C (55.7 ± 5.18%), followed closely by 38°C (55.4 ± 7.36%), while motility significantly decreased at 40°C (45.4 ± 5.66%). A similar trend was observed in progressive motility, which was highest at 34°C (55.5 ± 5.46%) and 38°C (54.7 ± 8.18%), but declined notably at 40°C (44.4 ± 8.84%), indicating reduced sperm movement efficiency at elevated temperatures.

Among the kinetic parameters, curvilinear velocity (VCL), representing the total distance travelled by sperm, was highest at 38°C (112.3 ± 8.33 µm/s), followed by 34°C (109.3 ± 4.66 µm/s), and was lowest at 40°C (102.6 ± 9.30 µm/s). This suggests that while 38°C supports sperm movement, a further increase in temperature reduces sperm velocity.

Similarly, average path velocity (VAP) and straight-line velocity (VSL), which indicate the actual path efficiency of sperm movement, were highest at 38°C (48.3 ± 3.21 µm/s and 35.3 ± 2.96 µm/s, respectively). Sperm at 34°C showed slightly lower VAP (47.4 ± 2.22 µm/s) and VSL (34.8 ± 2.47 µm/s), while at 40°C these values decreased to 44.8 ± 4.62 µm/s (VAP) and 31.9 ± 4.25 µm/s (VSL), reinforcing the negative impact of excessive heat on sperm velocity.

When assessing linearity (LIN), which measures the directness of sperm movement, the highest value was recorded at 34°C (32.1 ± 3.21%), followed by 40°C (27.5 ± 2.27%), while the lowest was at 38°C (21.2 ± 1.49%), indicating that sperm at this temperature exhibited a less linear movement pattern. However, straightness (STR), which represents the percentage of movement directed forward, was highest at 38°C (69.1 ± 2.04%), compared to 34°C (63.6 ± 3.67%) and 40°C (60.64 ± 4.39%).

The amplitude of lateral head displacement (ALH), an indicator of sperm head oscillation, was highest at 34°C (7.8 ± 0.92 µm) and decreased with increasing temperature (38°C: 7.2 ± 0.45 µm; 40°C: 6.4 ± 0.44 µm). The beat cross frequency (BCF), which represents the frequency of sperm tail oscillations, remained relatively stable across the temperatures, with the highest values recorded at 40°C (22.3 ± 1.80 Hz), compared to 38°C (21.6 ± 1.19 Hz) and 34°C (21.2 ± 1.54 Hz).

The wobble coefficient (WOB), which reflects the relationship between sperm velocity and head displacement, showed a slight decline at 40°C (40.6 ± 2.62%), while values at 38°C (43.8 ± 1.19%) and 34°C (43.3 ± 1.26%) were higher, indicating greater movement efficiency at lower temperatures.

Table 2. Temperature impact on canine sperm Kinetic features

Temperature/ Kintetic Parameters	34 °C	38 °C	40 °C	P
Motility (%)	55,7±5,18	55,4±7,36	45,4±5,66	NS
Progressive Motility (%)	55,5±5,46	54,7±8,18	44,4±8,84	NS
Curvilinear Velocity (um/s)	109,3±4,66	112,3±8,33	102,6±9,30	NS
Average Path Velocity (um/s)	47,4±2,22	48,3±3,21	44,8±4,62	NS
Straight Line Velocity (um/s)	32,8±2,47	35,3±2,96	31,9±4,25	NS
Linearity (%)	32,1±3,21	21,2±1,49	27,5±2,27	NS
Straightness (%)	63,6±3,67	69,1±2,04	60,6±4,39	NS
ALH (um)	7,8±0,92	7,2±0,45	6,4±0,44	NS
BCF (Hz)	21,2±1,54	21,6±1,19	22,3±1,80	NS
WOB (%)	43,3±1,26	43,8±1,19	40,6±2,62	NS

Sperm Membrane Integrity Assessment

The percentage of HOST-positive sperm, which indicates intact sperm membranes, significantly decreased with increasing temperature. Fresh samples initially contained 85.7 ± 6.18% HOST-positive sperm, but this percentage dropped to 65.4 ± 4.36% at 34°C, 67.4 ± 3.16% at 38°C, and declined further to 45.4 ± 4.61% at 40°C, suggesting that exposure to higher temperatures leads to a substantial reduction in membrane integrity.

Table 3. Temperature impact on canine HOST-Positive Sperm (%)

	HOST-Positive Sperm (%)
Start	85,7±6,18^{A}
34 °C	65,4±4,36^{B}
38 °C	67,4±3,16^{B}
40 °C	45,4±4,61^{C}

[a,b c] Significant of interaction effect on group averages indicated by different letters (P < 0.05).

Sperm Viability Assessment

Similarly, the percentage of live sperm (Live/Dead ratio) declined with increasing temperature. The fresh sample contained 88.1% live sperm (185/25). After 60 minutes of incubation, sperm survival rates were 69.8% (155/67) at 34°C, 66.6% (140/70) at 38°C, and significantly lower at 57.4% (120/89) at 40°C, demonstrating that sperm viability is negatively affected by higher temperatures.

Table 4. Temperature impact on canine HOST-Positive Sperm (%)

	% Sperm (Live/Dead)
Start (Fresh)	88,1 (185/25) [a]
34 °C	69,8 (155/67) [b]
38 °C	66,6 (140/70) [b]
40 °C	57,4 (120/89) [b]

[a,b] Significant of interaction effect on group averages indicated by different letters (P< 0.05

Sperm motility and concentration are critical parameters influencing fertilization success. While motility reflects the functional capacity of spermatozoa to reach and fertilize an oocyte, concentration provides insights into sperm retention and survival under different physiological conditions. Our study demonstrated that temperature significantly influences sperm concentration, with the highest sperm retention observed at 34°C, followed by 38°C, while the lowest concentration was recorded at 40°C. These findings suggest that excessive heat exposure negatively impacts sperm survival, possibly due to increased cellular stress and metabolic exhaustion. A comparison of our results with previous studies in canine and other mammalian species reveals a consistent trend where spermatozoa exhibit temperature-dependent survival patterns, which are closely linked to their metabolic activity and susceptibility to heat stress.

The observed higher sperm concentration at 34°C and 38°C aligns with findings from bovine and ovine sperm studies, which indicate that moderate temperatures (34°C–38°C) favor sperm retention and storage (García et al., 2021; Van de Hoek et al., 2022). In contrast, the sharp decline in sperm concentration at 40°C suggests that heat stress accelerates sperm degradation and loss, potentially through increased apoptosis, membrane damage, and oxidative stress. Studies on human and mice sperm have reported that capacitated sperm actively migrate toward 35–38°C temperatures (Pérez-Cerezales et al. 2018)

A possible explanation for this trend is that higher temperatures induce premature metabolic activation, leading to a faster depletion of ATP stores (Turner, 2006). Since spermatozoa rely on oxidative phosphorylation and glycolysis for energy production, prolonged exposure to excessive temperatures may overload mitochondrial activity, triggering a metabolic crisis that leads to reduced viability and increased sperm loss (Plessis et al., 2015). This hypothesis is supported by Van de Hoek et al. (2022), who demonstrated that higher metabolic demands at elevated temperatures compromise sperm endurance, resulting in reduced sperm numbers in the sample post-incubation.

The Nigrosin-Eosin staining and HOST results in our study provide additional insights into the temperature-dependent decline in sperm concentration. A significant drop in HOST-positive sperm at 40°C suggests that heat exposure weakens membrane integrity, making sperm more prone to lysis and loss. This is consistent with findings from Rickard et al. (2022), who reported that increased temperatures disrupt sperm lipid membranes, reducing their ability to withstand osmotic stress.

The reduced sperm retention at 40°C may also be attributed to heat-induced DNA fragmentation, which has been observed in thermally stressed spermatozoa in various species, including humans, bovines, and canines (Pérez-Cerezales et al., 2015). Thermal stress increases oxidative damage, leading to structural DNA instability, which in turn compromises sperm longevity. This mechanism could explain the significant sperm loss observed in our study at 40°C

Conclusion

This study highlights that temperature plays a crucial role in sperm concentration, motility, and viability. Sperm retention was highest at 34°C and 38°C, while exposure to 40°C resulted in significant sperm loss, likely due to heat-induced metabolic exhaustion, membrane instability, and oxidative stress. These findings align with previous research in bovine, human, and rodent sperm, supporting the conserved nature of temperature-dependent sperm survival mechanisms. The results have important implications for canine ART, emphasizing the need for precise temperature control in sperm handling and insemination protocols. Further research should focus on thermotactic-based sperm selection strategies to optimize fertilization success.

Acknowledgements

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