Farah Nurinda Amalia et al./Animal Production. 27 (3): 174-185, 2025
Accredited by Kemenristek Dikti No 32a/E/KPT/2017. ISSN 1411-2027

Post-Thaw Quality of Bali Cattle Semen in CEP Diluent: Optimizing Temperature and Duration

Farah Nurinda Amalia[1]*, Nur Ducha[1], Nur Qomariyah[1], Fardha Ad Durrun Nafis[2]

[1]Study Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Surabaya, Indonesia
[2]Artificial Insemination Center of Singosari, Singosari, Malang, Indonesia
***Corresponding author email: farahnurindaa2004@gmail.com**

Abstract. The thawing method is a crucial stage in the semen freezing process because it determines the quality of spermatozoa post-thawing. This study aims to evaluate the effect of thawing temperature and duration on the quality of frozen Bali cattle semen in CEP diluent. Semen was collected from a Bali bull at the Singosari Artificial Insemination Center, added to CEP diluent, and frozen. It was then thawed using three temperature variations (37°C, 30°C, and 25°C) and two durations (30 and 60 seconds) in a 3x2 factorial completely randomized design. Post-thawing evaluation included motility, viability, and membrane integrity analyzed using two-way ANOVA and Duncan's test. The results showed that thawing temperature significantly affected all parameters ($P<0.05$). A temperature of 37°C produced the highest motility, viability, and membrane integrity. Thawing duration significantly affected motility and membrane integrity, with the best results obtained at 30 seconds. There was no significant interaction between thawing temperature and duration, but the combination of 37°C and 30 seconds produced the best post-thawing motility (56.67±2.89). An optimal thawing combination was identified, with temperature and duration independently affecting sperm quality, offering practical guidelines for Bali cattle artificial insemination programs.

Keywords: thawing, temperature, duration, CEP diluent, Bali cattle

Abstrak. Metode *thawing* merupakan tahap krusial proses pembekuan semen karena menentukan kualitas spermatozoa pasca-thawing. Penelitian ini bertujuan mengevaluasi pengaruh suhu dan durasi thawing terhadap kualitas semen beku sapi Bali dalam pengencer CEP. Semen berasal dari satu ekor sapi Bali di Balai Inseminasi Buatan Singosari, ditambahkan pengencer CEP, dibekukan dan *thawing* menggunakan tiga variasi suhu (37°C, 30°C, dan 25°C) serta dua durasi (30 dan 60 detik) dalam rancangan acak lengkap faktorial 3×2. Evaluasi pasca-*thawing* meliputi motilitas, viabilitas dan integritas membran dianalisis menggunakan ANOVA dua arah dan uji Duncan. Hasil penelitian menunjukkan bahwa suhu *thawing* berpengaruh signifikan terhadap seluruh parameter ($P<0,05$). Suhu 37°C menghasilkan motilitas, viabilitas, dan integritas membran tertinggi. Durasi *thawing* berpengaruh signifikan terhadap motilitas dan integritas membran, dengan hasil terbaik 30 detik. Tidak terdapat interaksi signifikan antara suhu dan durasi thawing, namun kombinasi suhu 37°C durasi 30 detik menghasilkan motilitas pasca-*thawing* terbaik (56,67±2,89). Kombinasi *thawing* optimal berhasil diidentifikasi, dengan suhu dan durasi secara independen memengaruhi kualitas spermatozoa, sehingga memberikan panduan praktis untuk program inseminasi buatan sapi Bali.

Kata kunci: thawing, suhu, durasi, pengencer CEP, sapi Bali

Introduction

Bali cattle are a local Indonesian breed that is sought after by farmers for their dual function as workers and meat producers, with many advantages over other breeds (Fania et al., 2020). According to data from the Central Statistics Agency in 2023, Bali cattle dominate the beef cattle population in Indonesia at 38.59% (Aberth, 2024). Beef consumption is important for a balanced diet (Pighin et al., 2016). The demand for beef in Indonesia is increasing in line with population growth and income growth (Suryana et al., 2019; Syamsi et al., 2025). However, the Indonesian livestock industry has not been able to fully meet this demand, so efforts to increase livestock populations are needed.

Artificial insemination (AI) is an effective way to increase livestock population and quality because it produces large numbers of offspring with better quality and faster turnaround times (Elly et al., 2020). There are two semen storage techniques in AI, namely cooling (4-5°C) and freezing in liquid nitrogen (-196°C) (Bustani and

Baiee, 2021). Frozen semen is recommended because it has the advantage of lasting longer than liquid semen (Purnomo et al., 2019). A finding by Leibo et al. (1994) states that the motility of frozen bovine semen can remain normal even after being stored for 37 years. However, semen freezing carries the risk of quality deterioration due to ice crystal formation, drastic temperature changes, and osmotic pressure, which can damage membranes, reduce motility, and alter spermatozoa morphology (Rios and Botella, 2019).

One of the factors determining the success of the freezing process is the thawing method, which is the process of thawing frozen semen at physiological body temperature to reactivate spermatozoa cells (Permana et al., 2024). Thawing is the most crucial process in the cryopreservation protocol, because spermatozoa cells transition from a state of rest to sudden activation of cellular metabolism (Pezo et al., 2024). Improper thawing results in a significant decrease in motility and fertility potential (Koch et al., 2022). Additionally, it can cause a reduction in spermatozoa viability and membrane integrity due to cellular stress and ice crystal damage (Zenteno et al., 2023). According to Yılmaz et al. (2019), thawing at 37°C for 45 seconds and 70°C for 5 seconds produces the best quality, while thawing at 50°C for 15 seconds reduces spermatozoa viability. Meanwhile, research by Ramadhani et al. (2022) states that thawing at a low temperature (28°C) for 30 seconds produces the best motility.

The quality of frozen semen is also influenced by the addition of diluents. One diluent that can be used is Cauda Epididymal Plasma (CEP), which was discovered by Verbeckmoes et al. (2004) and modified by Ducha (2012) with specific protein content to maintain the spermatozoa membrane (Ducha, 2018). The use of CEP can improve post-thawing quality in Bali cattle with motility of 55.30±2.95^{b} (Fitri et al., 2025). The CEP diluent was also used by Rahayu and Ducha (2022) in their study on FH cattle, which mentioned thawing at 37°C for 5-10 seconds and produced a motility percentage of 27.18%-40.11%. The differences in spermatozoa characteristics between FH cattle and Bali cattle can affect semen quality results with different thawing methods, so it is necessary to test the semen quality of Bali cattle in relation to the thawing method in CEP diluent in order to achieve optimal post-thawing results.

Currently, there are no studies that specifically examine the effect of temperature variation and thawing duration on the quality of frozen Bali cattle semen, including motility, viability, and membrane integrity in CEP diluents. Therefore, this study aims to determine the optimal temperature variation and thawing duration by adjusting field conditions, which is expected to support the increase in the population and quality of local livestock in Indonesia.

Materials and Methods

CEP Diluent Construction

The CEP diluent (per litre) was prepared based on the formulation by Ducha (2018), with a composition 15 mmol NaCl; 7.0 mmol KCl; 3.0 mmol $CaCl_2(H_2O)_2$; 3.0 mmol $MgCl_2(H_2O)_6$; 11.9 mmol $NaHCO_3$; 8.0 mmol NaH_2PO_4; 20.0 mmol KH_2PO_4; 55 mmol fructose; 1.0 g sorbitol; 2.0 g Tris; 1000 IUI penicillin; 1 g streptomycin; 1 g citric acid; 42.6 mmol. BSA; 133.7 mmol and dissolved in aliquots using Otsuka sterile water in an Erlenmeyer. The solution was then sterilized using a 0.22 µm Millipore membrane, and then 20% chicken egg yolk was added and homogenized again. The entire procedure was performed under sterile conditions beside a Bunsen burner. The solution was left for 2-4 days in a refrigerator (4-5°C) until a supernatant formed, which was then used as a diluent.

Semen Collection and Dilution

Fresh semen was collected from a superior Bali bull named Sanur, owned by the Singosari

Artificial Insemination Center (BBIB), using the Artificial Vagina (AV) method. The Bali bull is 13 years old and weighs 592 kg. The bull is one of the healthy Bali bulls that undergoes semen collection once a week. The semen used in this study was collected once from the same bull in each repetition, and no samples were pooled.

Fresh semen that has been collected is then taken to the laboratory for macroscopic examination, which includes color, pH, odor, volume, consistency, and concentration, as well as microscopic examination, which includes motility, viability, and membrane integrity. The percentage of progressive motility of spermatozoa in fresh semen must be at least 70% (SNI, 2024).

The dilution stages were carried out based on the method described by Rozi and Ducha (2021), which was divided into three stages, namely A1, A2, and B. Dilutions A1 and A2 were carried out at a temperature of 37°C in a water bath, which was then gradually lowered to a temperature of 5°C. Meanwhile, dilution B was carried out in a cool tube at a temperature of 5°C with the addition of cryoprotectants such as glycerol. The volume ratio of the diluent added in A1 to the volume of fresh semen was 1:1 (Fitri et al., 2025). Liquid semen or diluted A1 stored at a temperature of 3-5°C is used for diluting A2. The volume of diluent used in stage A2 was calculated using the following formula:

$$\text{V.total} = \frac{\text{V. semen x semen concentration } (10^6)}{(\frac{25x10^6}{0.25})}$$

$$\text{V. A2} = \text{V. total} - (\text{V. semen} + \text{V. A1} + \text{V. B})$$

Each insemination dose in a 0.25 mL straw contains approximately 25 × 10^6 progressive motile spermatozoa (SNI, 2024). The diluted A2 semen is stored in a refrigerator at a temperature of 3-5℃ for approximately 18 hours. The next step was to add half of the total volume of diluent B (diluent A and 13% glycerol) (Rahayu and Ducha, 2022). The volume of diluent B can be calculated using the following formula:

$$\text{V. B} = \frac{\text{V. total}}{2}$$

Semen evaluation before freezing (BF) is performed before the freezing process. The evaluation was carried out by observing the motility, viability, and membrane integrity of spermatozoa using a 400x magnification microscope. The percentage of progressive motility of spermatozoa that can proceed to the freezing process has a minimum requirement of 55% (To'aloh et al., 2023).

Freezing Process

The semen freezing method was carried out over 2 days in several stages. The first cooling (dilution A1) was placed in a water jacket in a refrigerator until the temperature dropped to 3-5°C for 4-6 hours, followed by a second cooling (dilution A2) for 18 hours. The equilibration stage was carried out after the addition of cryoprotectant (glycerol) through dilution B at a temperature of 3-5°C for 2-4 hours. The semen is then packaged into tubes (0.25 ml) that have been sterilized with UV light for 15 minutes using an automatic filling and sealing device, then sealed. The straws were stored at 3-5°C and then placed on the surface of liquid nitrogen approximately 6 cm away for 15 minutes as a pre-freezing stage (Syaifullah et al., 2022). The pre-freezing stage was carried out to rapidly lower the temperature from 4°C to -140°C. The straws were then placed in goblets and stored in a container filled with liquid nitrogen at a temperature of -196°C.

Thawing Method

Thawing was carried out by removing the straws containing frozen semen from the liquid nitrogen container and immediately immersing them in warm water (Marlize et al., 2021). The water was placed in a water bath with 3 temperature variations (37°C, 30°C, 25°C) and 2 duration variations (30 and 60 seconds). These temperature variations were chosen because they correspond to thawing in laboratories,

which is generally in line with the physiological temperature of cows (37°C), and to field conditions, which generally use ambient water temperatures (30°C and 25°C). Meanwhile, thawing durations of 30 and 60 seconds are used to determine whether a longer or shorter thawing time is optimal without causing thermal damage to the spermatozoa. Both ends of the straw containing thawed semen are cut to transfer the semen into a 1.5 ml microtube.

Post-Thawing Evaluation

Post-thawing semen evaluation is performed by testing the motility, viability, and membrane integrity of spermatozoa. Individual motility is observed by placing 10 µL of semen on a glass slide, followed by examination under a microscope at 400x magnification. Spermatozoa motility testing can be performed by placing 10 µL of semen on a glass slide and observing it under a microscope at 400x magnification (Ducha et al., 2023). The sample is then covered with a cover glass and placed on a slide heater at 37°C. The percentage of progressive motility of spermatozoa after thawing has a minimum requirement of 40% (SNI, 2024).

Viability testing was performed by observing the object glass that had been dripped with semen and eosin nigrosin dye (1:2), made into a thin smear, and counted per 200 spermatozoa per sample observed using a 400x magnification microscope (Ducha et al., 2025). The percentage of post-thawing spermatozoa viability has a minimum requirement of 50% (Ducha, 2018). Viability was calculated as:

$$\%\text{Viability} = \frac{\text{total of live spermatozoa}}{total\ spermatozoa\ (live\ and\ dead)} \text{ x } 100\%$$

Membrane integrity testing was performed with semen homogenized in HOST solution (1.3 g fructose and 0.7 g trisodium citrate/100 ml distilled water at a ratio of 1:10), incubated at 37°C for 30 minutes, made into thin smears on glass slides, counted per 200 spermatozoa per sample under a microscope at 400x magnification and had a minimum percentage of 55% post-thawing (Fitri et al., 2025). The membrane integrity calculation was:

$$\%\text{Membrane Integrity} = \frac{\text{total of coiled tail spermatozoa}}{\text{total spermatozoa}} \text{ x } 100\%$$

Research Design and Variables

This study was an experiment using a 3x2 factorial completely randomized design. Each treatment was repeated three times. The three repetitions were laboratory tests of the same semen and bull samples, not repeated semen collection. There are two factors in this study, namely temperature and thawing duration. This study has three interrelated treatment variables, namely the independent variables of thawing temperature (37°C, 30°C, 25°C) and thawing duration (30 and 60 seconds). The dependent variables include frozen semen quality, which is assessed based on motility, viability, and spermatozoa membrane integrity. The control variables include the type of diluent, the type of cow, and the volume of semen.

Data Analysis

Data analysis was performed using SPSS Statistics 23 for Windows. The analysis stages included a homogeneity of variance test to ensure that the assumptions of variance analysis were met. A two-way ANOVA test was then conducted to determine the effect of temperature and thawing duration on semen quality. The analysis results showed a significant difference at a significance level of 0.05 ($P<0.05$), so the analysis was continued with a Duncan post hoc test to identify the best treatment combination. The analysis results were then presented in tabular form to facilitate data interpretation.

Results and Discussion

Fresh semen collected directly is taken to the laboratory for macroscopic and microscopic evaluation. Macroscopic and microscopic observations aim to assess the quality of fresh

semen from Bali bulls through several parameters, and if the semen is categorized as acceptable, it is then subjected to microscopic examination (Prastiya et al., 2024). The macroscopic parameters observed include color, pH, consistency, and volume, while the microscopic evaluation includes concentration, abnormalities, motility, viability, and membrane integrity. The motility, viability, and membrane integrity evaluations are averaged, and the standard deviation is calculated from three repetitions. The results of the fresh semen quality test can be seen in Table 1.

Based on the data in Table 1, the evaluation results of the quality of fresh semen from Bali cattle can be considered good. The motility of fresh semen has a minimum value of 70% to proceed to freezing (Purnawan et al., 2025). Fresh bovine semen viability is considered good if the percentage is ≥70%, while membrane integrity of 73.7% is considered high (Ducha, 2018). The data in Table 1 meet the minimum requirements for proceeding to the freezing process. Evaluation before freezing and after thawing is then carried out after evaluation of fresh semen. The evaluation before freezing was necessary to determine the suitability of the semen before freezing and the effect of the additive content in maintaining spermatozoa quality during the freezing process (Masyitoh et al., 2018). This evaluation aimed to ensure the quality of the spermatozoa after the addition of glycerol and CEP diluent. Post-thawing evaluation is conducted afterwards to analyze the quality of spermatozoa after freezing and thawing. Both evaluations are conducted using three parameters, namely motility, viability, and membrane integrity. The results of the pre-freezing and post-thawing motility evaluations can be seen in Table 2.

The results of the two-way ANOVA analysis support the results in Table 2, that the factors of temperature (P=0.007) and duration (P=0.046) have a significant effect on motility, but the interaction of the two factors does not have a significant effect on motility (P>0.05) (P=0.490). This indicates that temperature and thawing duration can have independent effects.

Table 1. Macroscopic and microscopic evaluation of fresh semen from a Bali bull

Parameters	Mean±Standard Deviation
Color	Milky white
pH	6.70±0.10
Consistency	Thick
Volume (ml)	5.67±1.15
Concentration (million/ml)	997.33±97.27
Abnormality (%)	9.53±0.40
Progressive Motility (%)	73.33±2.89
Viability (%)	88.50±1.02
Membrane Integrity (%)	82.16±1.87

Table 2. The mean±standard deviation of frozen semen motility in a Bali bull at various temperatures and thawing durations

Temperature (°C)	Duration (seconds)	Before Freezing	Post-Thawing
37	30	66.67±2.89^{b}	56.67±2.89^{b}
37	60	58.33±5.77^{ab}	51.67±2.89^{b}
30	30	60.00±5.00^{ab}	51.67±2.89^{ab}
30	60	61.67±2.89^{b}	48.33±2.89^{b}
25	30	58.33±2.89^{a}	46.67±2.89^{a}
25	60	51.67±2.89^{a}	41.67±2.89^{a}

Notes: Values with different superscript letters (^{a,b,c}) on the same line indicate significant differences (P<0.05), while values with the same letters are not significantly different.

However, even though there was no interaction between temperature and duration, the best combination was produced by the treatment of 37°C temperature and 30 seconds duration (56.67±2.89b), while the lowest was at 25°C temperature and 60 seconds duration (41.67±2.89[a]). The standard deviation values for motility were relatively small, and the motility data between replicates were quite consistent.

Motility is the progressive movement of spermatozoa cells that move actively in a straight line or in large circles (Hook and Fisher, 2020). Based on the research results, although there was no interaction between temperature and thawing duration, higher thawing temperatures resulted in better motility, but excessively long thawing durations could reduce motility. According to Koch et al. (2022), in recent years, several studies have shown that faster thawing at higher temperatures within a short time interval results in better semen quality and longer post-thawing viability compared to prolonged thawing.

Research by DeJarnette and Marshall (2005) suggests that thawing for longer periods at lower temperatures reduces fertilization potential by damaging the integrity of the acrosome and spermatozoa motility. Excessively long thawing durations can increase lactic acid production due to increased overall metabolic activity, which has a toxic effect on spermatozoa concentration, slowing sperm motility and accelerating death (Ramadhani et al., 2022).

A minimum post-thawing motility percentage of 40% according to SNI is one of the quality standards for frozen semen (Permana et al., 2024). Based on the data in Table 2, all treatments had results above the minimum requirement and passed for distribution. Therefore, if thawing is done outside of laboratory control under field conditions, tap water at 30°C and 25°C can be used with the appropriate thawing duration.

The results of the two-way ANOVA analysis showed that the temperature factor had a significant effect on viability (P=0.023), while the thawing duration factor (P=0.073) and the interaction between the temperature and duration factors did not show a significant effect (P>0.05) (P=0.972). However, according to the results of the study by Nguyen et al. (2023), rapid thawing can increase viability and produce the best quality compared to slower thawing. The best post-thawing viability was produced by the 37°C temperature treatment with a duration of 30 seconds, at 79.30±1.70[b], while the lowest viability was produced by the 25°C temperature treatment with a duration of 60 seconds, at 67.22±0.61[a]. The standard deviation value for viability was relatively small, the viability data between replicates were quite consistent, and the differences in the data were due to the effect of the treatment.

Although the interaction of the two factors was not significant, the best combination of thawing methods to maintain the quality of frozen Bali cattle semen was at a temperature of 37°C for 30 seconds. According to Aksu et al. (2024), previous studies have shown that thawing at moderate temperatures is more effective in maintaining higher spermatozoa viability compared to extreme temperatures. Viability is a parameter for determining live and dead spermatozoa using the eosin-nigrosin staining method. Eosin-nigrosin dye is conventionally used as a differential stain to assess the scale of live and dead spermatozoa (Tanga et al., 2021).

The data in Table 3 show post-thawing viability above the minimum standard because the thawing temperature used was within the normal range and not extreme. The thawing method did not significantly affect spermatozoa viability. This may be because the variations in thawing temperature and duration did not cause extreme changes in osmotic pressure, thereby maintaining stable and undisturbed spermatozoa membrane permeability (Fitriana et al., 2025; Surjamah et al., 2024).

Table 3. The mean±standard deviation of frozen semen viability in a Bali bull with various thawing temperatures and durations

Temperature (°C)	Duration (seconds)	Before Freezing	Post-Thawing
37	30	85.94±1.07^{b}	79.30±1.70^{b}
37	60	83.83±1.81^{b}	75.00±0.40^{c}
30	30	85.33±1.67^{b}	77.08±1.21^{b}
30	60	82.23±1.85^{ab}	73.47±0.82^{b}
25	30	80.72±1.59^{a}	71.95±1.12^{a}
25	60	80.10±1.08^{a}	67.22±0.61^{a}

Notes: Values with different superscript letters (^{a,b,c}) on the same line indicate significant differences (P<0.05), while values with the same letters are not significantly different.

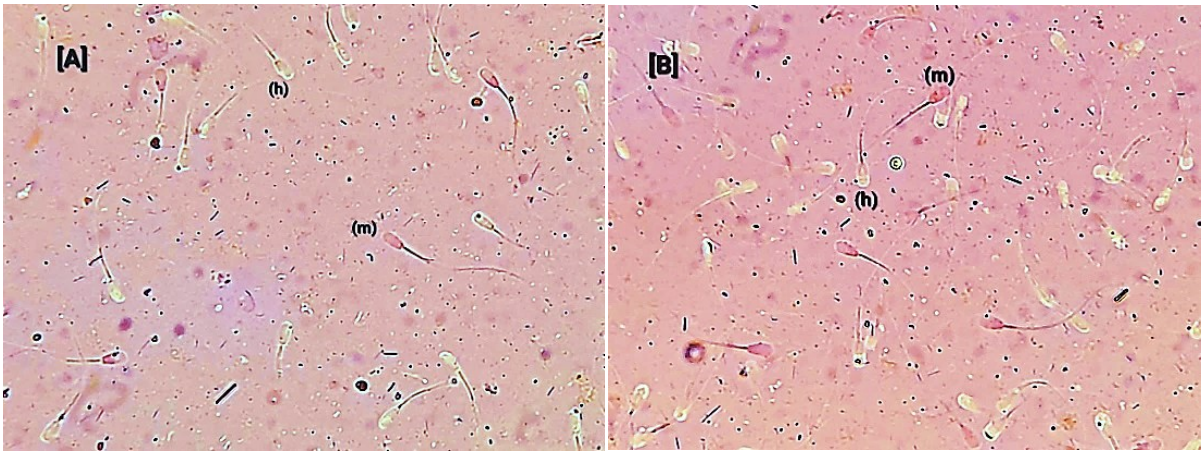

Figure 1. Post-thawing viability of Bali cattle enlarged 400x. Notes: [A] 37°C 30s; [B] 25°C 60s; (h) live spermatozoa; (m) dead spermatozoa

Therefore, based on the data in Table 3, the percentage of post-thawing viability is classified as good and acceptable. This is in line with the viability of Bali cattle post-thawing in CEP diluent found in the study by Fitri et al. (2025), which showed that a percentage of 71.26% is classified as good.

The study by Malinda et al. (2021) also found that the interaction between temperature and thawing time was not significantly different, but too long a thawing duration can reduce spermatozoa viability quality because the speed of change during thawing affects the critical phase, which can be passed quickly due to a reduction in pressure on the spermatozoa, resulting in a higher percentage of live spermatozoa.

The results of post-thawing viability observations using eosin-nigrosin staining can be seen in Figure 1. According to Nizam et al. (2025), live spermatozoa are characterized by white heads (uncolored), while dead spermatozoa are characterized by heads that absorb a purplish-pink color. The percentage is determined by counting a total of 200 spermatozoa per stained field of view. The results show that at a temperature of 37°C and a duration of 30 seconds, most spermatozoa are still alive with the highest post-thawing viability percentage.

The results show that at a temperature of 37°C for 30 seconds, most spermatozoa did not absorb the dye, indicating that they were still alive, with the highest post-thawing viability percentage. Meanwhile, at a temperature of 25°C for 60 seconds, almost all spermatozoa cells died and turned purplish pink.

The results of the two-way ANOVA analysis show that temperature has a significant effect on membrane integrity (P=0.000), duration has a significant effect on membrane integrity (P=0.002), while the interaction between temperature and duration has no significant effect on membrane integrity (P>0.05) (0.444).

Table 4. The mean±standard deviation of frozen semen membrane integrity in a Bali bull with various thawing temperatures and durations

Temperature (°C)	Duration (seconds)	Before Freezing	Post-Thawing
37	30	78.64±1.38^{b}	77.18±1.14^{c}
37	60	77.16±0.80^{b}	72.25±1.12^{b}
30	30	77.98±1.10^{b}	73.62±0.85^{b}
30	60	73.23±1.96^{a}	70.53±1.74^{b}
25	30	71.63±1.79^{a}	68.43±1.66^{a}
25	60	71.24±1.28^{a}	66.06±0.53^{a}

Notes: Values with different superscript letters (^{a,b,c}) on the same line indicate significant differences (P<0.05), while values with the same letters are not significantly different.

Similar to motility, temperature and thawing duration can have independent effects without any interaction between them. However, the best combination of post-thawing treatments was a temperature of 37°C and a duration of 30 seconds, resulting in 77.18±1.14c 77.18±1.14c. Meanwhile, thawing at a temperature of 25°C for 60 seconds reduced the integrity of spermatozoa membranes after thawing to 66.06±0.53^{a} (See Table 4).

Temperature and duration factors have a significant effect on membrane integrity, while the interaction between the two factors is not significant. Temperature affects the rate of crystal melting, and duration affects the length of exposure to the thawing medium, which can trigger osmotic imbalance (Umirbaeva et al., 2024). Based on the research results, higher thawing temperatures tend to maintain spermatozoa membrane integrity, but excessive thawing duration can reduce spermatozoa quality. Membrane integrity refers to the condition of the plasma membrane integrity in spermatozoa. These results are consistent with recommendations for rapid thawing at a temperature range of 35–37 °C for bovine semen, as this minimizes ice recrystallization and osmotic stress that damages the plasma membrane (Goshme et al., 2021; Solís et al., 2024).

Biologically, post-thawing membrane integrity is largely determined by the susceptibility of membrane lipids to oxidation and osmotic fluctuations during the thawing phase. Increasing the thawing temperature appropriately helps to pass through the critical temperature zone more quickly, thereby suppressing ice recrystallization and reducing membrane damage, while excessive thawing duration increases ROS and lipid peroxidation, which disrupt membrane fluidity (Tanga et al., 2021). In addition, research by Nagy et al. (2004) found that the longer semen is left during thawing without optimal handling, the more cells experience plasma membrane damage.

The results of observations of post-thawing membrane integrity after incubation in HOST solution can be seen in Figure 2. Spermatozoa with intact membranes are indicated by curled tails, while spermatozoa with non-intact membranes have straight tails. The results show that at 37°C for 30 seconds, most spermatozoa membranes were still intact and curled, with the highest percentage of membrane integrity after thawing. Meanwhile, thawing at 25°C for 60 seconds showed more straight tails than curled ones due to low membrane integrity.

Based on the results of the study, the effect of temperature has a greater impact on spermatozoa quality than duration. There is no significant effect of the interaction between the two factors, and each factor can have an independent effect. However, the results show that a higher thawing temperature with a shorter duration is the optimal variation for maintaining frozen semen quality. Increasing the temperature can result in good motility, viability, and membrane integrity, but these good results can decrease if the thawing duration is too long.

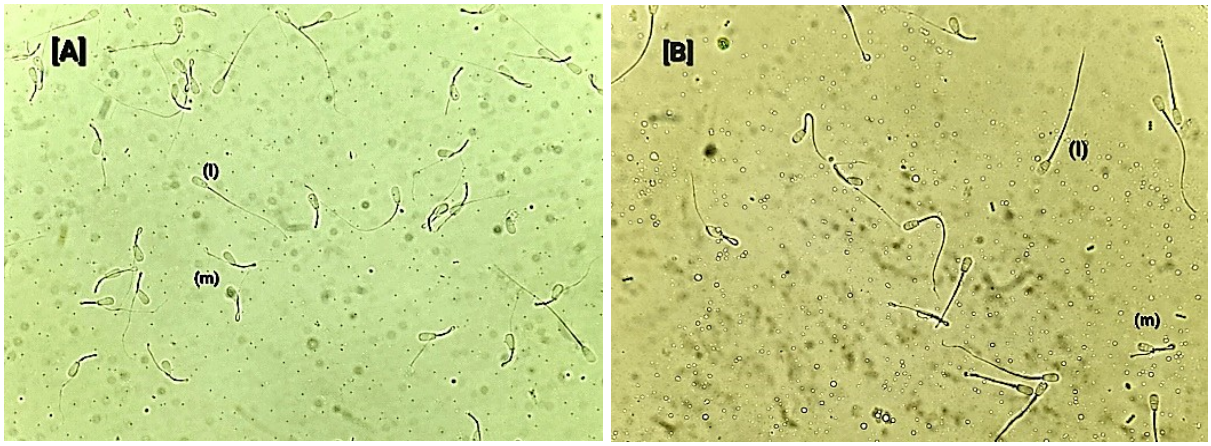

Figure 2. Membrane integrity after thawing of Bali cattle at 400x magnification. Notes: [A] 37°C 30s; [B] 25°C 60s; (m) spermatozoa tail coiled; (l) spermatozoa tail straight

According to research by Solis et al. (2024), variations in temperature and time have an initial effect on semen quality. The semen was incubated in the same environment after thawing, so that the difference between the interactions of the two factors became insignificant and the two factors tended to have independent effects. The results of this study also suggest that there is no consistent significant interaction between temperature and time factors in predicting spermatozoa motility resistance after thawing. Chandler et al. (1984) found that several variations in temperature and time produced equivalent thawing rates, so there was no significant interaction between the two factors on the quality of frozen semen after thawing as long as it remained within the usual operational range. The range of temperature variations (37°C, 30°C, 25°C) and thawing duration (30 and 60 seconds) used in this study were not significantly different and were within the normal range. The highest temperature used in the study was 37°C, which is recommended by SNI (2024) for thawing frozen semen. SNI (2024) states that thawing should be carried out at a temperature of 37°C to 38°C for 30 seconds.

Conclusions

Based on the results of the study, the thawing process is a crucial factor that determines the quality of frozen Bali cattle semen in CEP diluent. The variations in thawing temperature and duration that were tested showed that both factors independently affect spermatozoa motility, viability, and membrane integrity, without any significant interaction between the two factors. The optimal thawing treatment combination was successfully identified within the range of temperatures and durations studied. These findings provide practical guidelines that can be applied in the field to improve the effectiveness of artificial insemination programs and support the development of the Bali cattle population as a local genetic resource.

References

Aberth WAVP. 2024. Simak Jenis Sapi Potong di Indonesia: Sapi Bali Terbanyak. Goodstats. https://data.goodstats.id/statistic/simak-jenis-sapi-potong-di-indonesia-sapi-bali-terbanyak-8LVc2. (accessed: September 16, 2025).

Aksu EH, EY Kurtuluş, and B Aktürk. 2024. The Effects of Different Thawing Procedures on Spermatological Parameters of Frozen Bull Sperm. *Turkish Journal of Agriculture - Food Science and Technology*, 12(6): 1068–1071.

Bustani GS and FH Baiee. 2021. Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Veterinary World*, 14(5): 1220–1233.

Chandler JE, CF Ruiz, RW Adkinson, and KL Koonce. 1984. Relationship between final temperature,

thaw rate, and quality of bovine semen. *Journal of Dairy Science*, 67(8): 1806–1812.

De Jarnette JM and CE Marshall. 2005. Straw-thawing method interacts with sire and extender to influence sperm motility and conception rates of dairy cows*. Journal of Dairy Science*, 88 : 3868–3875.

Ducha N. 2018. The test about blood serum capabilities in maintaining the quality of bull spermatozoa during storage in CEP diluent at refrigerator temperature. *IOP Conference Series: Earth and Environmental Science,* 130: 012043.

Ducha N, T Susilawati, Aulanni'am, S Wahyuningsih and M Pangestu. 2012. Ultrastructure and fertilizing ability of Limousin bull sperm after storage in CEP-2 extender with and without egg yolk. *Pakistan Journal of Biological Sciences*, 15(20): 979–985.

Ducha N, L Lisdiana, G Trimulyono, FI Muhaimin, NNM Yusof, N Samsulrizal, R Dasiman, A Fudhaili, GRA Pramesti and JD Rahayu. 2023. Morphology and reproducivity profiling of male Senduro goats based on age differences. *Online Journal of Animal and Feed Research*, 13(6): 460–466.

Ducha N, F Isnawati, FI Muhaimin, SD Prastyaningtias, FAD Nafis and R Fitri. 2025. Sperm quality profile of Bali bull as local Indonesian cattle in liquid storage in different diluents with the addition of synthetic antioxidants. *BIO Web of Conferences,* 162 : 00012.

Elly FH, Z Poli, CL Kaunang, A Lomboan, AHS Salendu, and R Ningalo. 2020. Introduction prospect of artificial insemination technology in supporting beef cattle development. *International Journal of Environment, Agriculture and Biotechnology,* 5(6): 1609–1615.

Fania B, IGNB Trilaksana, dan IK Puja. 2020. Keberhasilan inseminasi buatan pada sapi Bali di Kecamatan Mengwi, Badung, Bali. *Indonesia Medicus Veterinus*, 9(2): 177–186.

Fitri R, N Ducha, and D Retnowulan. 2025. Effects of adding glutathione to CEP diluent on post-thawing sperm quality of Bali cattle. *LenteraBio,* 14(1): 63–71.

Fitriana SB, NA Maghfiroh, AN Baity, DFFF Diatmono, KD Prihantoko, S Bintara, and DT Widayati. 2025. Effect of different thawing methods on frozen semen characteristics and DNA damage of Indonesian Simmental bull. *Pakistan Journal of Agricultural Research*, 38(1): 8–18.

Goshme, S, T Asfaw, C Demiss, and S Besufekad. 2021. Evaluation of motility and morphology of frozen bull semen under different thawing methods used for artificial insemination in North Shewa zone, Ethiopia. Heliyon, 7(10).

Hook KA and HS Fisher. 2020. Methodological considerations for examining the relationship between sperm morphology and motility. *Molecular Reproduction and Development*, 87(6): 633–649.

Koch J, LP Weber, M Heppelmann, F Freise, M Klingelmann, and L Bachmann. 2022. Effect of different thawing methods for frozen bull semen and additional factors on the conception rate of dairy cows in artificial insemination. *Animals*, 12(18): 2330.

Leibo S, M Semple, and T Kroetsch. 1994. In vitro fertilization of oocytes by 37-year-old cryopreserved bovine spermatozoa. *Theriogenology*, 42(1): 1257–1262.

Malinda D, H Santoso, and H Latuconsina. 2021. Analisis viabilitas spermatozoa sapi Friesian Holstein post thawing semen beku dengan pengaruh suhu dan lama waktu thawing berbeda. *Jurnal Ilmiah Biosaintropis,* 6(2): 46–51.

Marlize S, TM Hine, and WM Nalley. 2021. Pengaruh waktu ekuilibrasi terhadap kualitas semen beku babi Landrace dalam pengencer Durasperm termodifikasi. *Jurnal Nukleus Peternakan,* 8(2): 150–160.

Masyitoh H, TW Suprayogi, RN Praja, P Srianto, SP Madyawati, and AL Saputro. 2018. Persentase motilitas dan viabilitas spermatozoa kambing Sapera dalam pengencer Tris kuning telur dan susu skim kuning telur before freezing. *Jurnal Medik Veteriner*, 1(3): 105–112.

Nagy S, T Hallap, A Johannisson, and H Rodriguez-Martinez. 2004. Changes in plasma membrane and acrosome integrity of frozen-thawed bovine spermatozoa during a 4 h incubation as measured by multicolor flow cytometry. *Animal Reproduction Science,* 80(3–4): 225–235.

Nguyen HT, SQ Do, R Athurupana, T Wakai, and H Funahashi. 2023. Rapid thawing of frozen bull

spermatozoa by transient exposure to 70 °C improves the viability, motility and mitochondrial health. *Animal Reproduction*, 20(3): e20220127.

Nizam MY, M Selçuk, and B Esin. 2025. Which is more effective in thawing frozen rooster sperm: varying temperature or duration? *Frontiers in Veterinary Science*, 12: 1534638.

Permana RGS, SA Puspitadesy, A Nareswari, and S Ediyono. 2024. Influence of method thawing to success artificial insemination of cows. *International Journal of Life Science and Agriculture Research,* 3(4): 230–237.

Pezo F, MJ Contreras, F Zambrano, P Uribe, J Risopatron, AFC de Andrade, M Yeste, and R Sánchez.

2024. Thawing of cryopreserved sperm from domestic animals: impact of temperature, time, and addition of molecules to thawing/insemination medium. *Animal Reproduction Science*, 268: 107572.

Pighin D, A Pazos, V Chamorro, F Paschetta, S Cunzolo, F Godoy, V Messina, A Pordomingo, and G Grigioni. 2016. A contribution of beef to human health: a review of the role of the animal production systems. *The Scientific World Journal,* 2016: 8681491.

Prastiya RA, T Sardjito, AL Saputro, DS Budi, SI Maxdhameta, E Sulistiyawati, D Sulistyowati, A Amaliya, SMS Sasi, and AN Haryuni. 2024. Quality and kinematic characteristics of Bali bulls frozen semen with purified green tea extract epigallocatechin-3-gallate antioxidant addition in diluent. *Open Veterinary Journal,* 14(8): 2040–2048.

Purnawan AB, R Rimayanti, S Susilowati, E Safitri, T Hernawati, I Mustofa, ZNA Rosyada, AO Akintunde, and AR Khairullah. 2025. Kinematic and morphological evaluation of frozen Friesian Holstein semen stored for 33, 30, 27, and 24 years. *Open Veterinary Journal,* 15(4): 1747–1756.

Purnomo M, H Supriyono, and Y Nasata. 2019. Pengaruh suhu dan lama thawing terhadap kualitas spermatozoa pada straw sapi Bali. *Stock Peternakan*, 2: 1–16.

Rahayu JD and N Ducha. 2022. Pengaruh air tebu sebagai kandidat pengganti fruktosa dalam pengencer CEP terhadap kualitas spermatozoa sapi Friesian Holstein selama penyimpanan beku. *Jurnal Ilmiah Peternakan Terpadu*, 10(2): 209–231.

Ramadhani N, A Rachmawati, AB Purnawan, T Susilowati, and APA Yekti. 2022. Pengaruh lama thawing dengan air dingin pada semen beku sapi Peranakan Ongole terhadap kualitas semen. *Livestock and Animal Research*, 20(3): 321–329.

Rios AP, and IM Botella. 2019. Causes and impact of cryopreservation-associated damage on different parameters of human spermatozoa and its clinical impact. *EMJ Repro Health*, 5(1): 100–109.

Rozi MFF, and N Ducha. 2021. The effect of glycerol concentration in the Tris-soya diluent on the motility of Boer goat spermatozoa before and after freezing. *LenteraBio,* 9(1): 12–16.

SNI (Standar Nasional Indonesia).2024. Penetapan SNI 4869-1:2024 Semen Beku – Bagian 1: Sapi.

Solís JM, F Sevilla, MA Silvestre, I Araya-Zúñiga, ERS Roldan, A Saborío-Montero, and A Valverde.

2024. Effect of thawing procedure and thermo-resistance test on sperm motility and kinematics patterns in two bovine breeds. *Animals*, 14(19): 2768.

Surjamah, R Somanjaya, and D Widianingrum. 2024. Spermatozoa quality of Pasundan cattle frozen semen at various thawing temperatures and durations. *Jurnal Sains Peternakan Indonesia*, 19(3): 132–138.

Suryana EA, D Martianto, and YF Baliwati. 2019. Pola konsumsi dan permintaan pangan sumber protein hewani di Provinsi Nusa Tenggara Barat dan Nusa Tenggara Timur. *Analisis Kebijakan Pertanian*, 17(1): 1–12.

Syaifullah F, K Khaeruddin, A Agus, A Mangalisu, R Faridah, S Farida, and J Junaedi. 2022. Viabilitas post ekuilibrasi dan recovery rate spermatozoa sapi Simmental dengan penambahan laktosa dan sukrosa dalam pengencer Andromed. *Jurnal Ilmu Peternakan (JANHUS),* 7(1): 38–48.

Syamsi, AN, N Kusrianty, KA Sahiman, YNN Ardilla, EP Pinandita, and P Utami. 2025. Rasionalitas Ketercapaian Swasembada Daging 2026 Berdasarkan Analisis Tren dan Peramalan Produksi Daging Sapi-Kerbau Berbasis Data Badan Pusat Statistik. Journal of Livestock Science and Production, 9(1), 1-13.

Tanga BM, AY Qamar, S Raza, S Bang, X Fang, K Yoon, and J Cho. 2021. Semen evaluation: methodological advancements in sperm quality-specific fertility assessment – a review. *Animal Bioscience*, 34(8): 1253–1270.

To'aloh N, NJ Mubarakati, and GE Jayanti. 2023. Analisis motilitas spermatozoa sebelum dan sesudah pembekuan pada sapi Peranakan Ongole (Bos indicus) di BBIB Singosari Malang. *JCS: Journal of Comprehensive Science,* 2(5):1031-1038.

Umirbaeva, A, A Kurenkov, A Makhanbetova, B Seisenov, IA Vorobjev, and NS Barteneva. 2024. Systematic review and meta-analysis of cryopreserved bovine sperm assessment: harnessing imaging flow cytometry for multi-parametric analysis. Frontiers in Veterinary Science, 11, 1371586.

Yılmaz E, K Ak and A Baran. 2019. Effect of different thawing time and high temperature on frozen-thawed bull semen traits. *Journal of Animal and Veterinary Advances*, 18: 239–245.

Zenteno ES, B Rojano, and GR Betancur. 2023. Influence of thawing temperature on sperm motility, structure, and metabolism of frozen bovine semen. *Ciencia Rural,* 53(3).