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Manuka honey in skim milk- or egg yolk-based extenders impacts stallion chilled spermatozoa characteristics, sperm-free enzymatic activity and lipid peroxidation

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ABSTRACT

To investigate impact of manuka honey (MH) on chilled spermatozoa characteristics, sperm-free enzymatic activity and lipid peroxidation, stallion semen samples (n=48) were diluted with Kenney (Skim milk-based, Ken) and Dimitropoulos (Egg yolk-based, Dimitri) extenders supplemented with MH at 0, 1, 2, and 4 %, and maintained at 4 °C for 72 hours. Progressive motility, livability, and morphology of sperm along with, acid phosphatase (ACP), aspartate aminotransferase (AST), acrosin, catalase (CAT), superoxide dismutase, glutathione peroxidase, and malondialdehyde (MDA) of semen were assessed at 12-hour intervals. Results showed that MH (2%) in Ken improved livability at 24-72 hrs and in Dimitri extenders enhanced motility at 72 hrs. ACP decreased with MH 2% in Ken at 0 (P= 0.007) and 12 hrs (P= 0.03), and in Dimitri at 12 (P= 0.001), 36, 48 (P= 0.001), 60 and 72 hours (P= 0.002). MH (1-4%) in both extenders markedly (P< 0.001) reduced AST and increased CAT up to 72 hrs, with 2% MH showing a profound effect. The acrosin enzyme markedly (P< 0.05) decreased in 2% MH-Ken group during the storage period. MH 2%-Ken at 0 and 12 hrs (P= 0.007 and 0.03) and Dimitri up to 72 hrs showed decreased (P< 0.001) MDA levels. MH with Ken/Dimitri extenders had a powerful effect in improving stallion chilled semen properties. MH-Ken was superior in improving semen characteristics, antioxidant defense, and potential fertilizing capacity while MH-Dimitri was good at maintaining wall integrity and shielding lipid peroxidation.

Keywords: Antioxidant, Dimitropoulos extender, Kenney extender, Manuka honey, Stallion semen

Stallion sperm are more susceptible to cold shock than sperm of other species due to high (18.2%) plasma membrane concentration of arachidonic acid (Chow *et al.* 1986), and cooled semen shows a declining livability after 24 hours of storage (LeFrapper *et al.* 2010). The extenders protect sperm against temperature changes through its contents of egg yolk or milk/milk products (Bergeron *et al.* 2007). Kenney's extender consists of skimmed milk added to glucose, electrolytes and antibiotics (Katila *et al.* 1997) and has been proven to maintain motility, vigor, and fertility of stallion semen after 24 hours of cold storage (Pugliesi *et al.* 2012). Dimitropoulos extender is an egg-based extender that maintains high progressive motility of stallion spermatozoa at 24-48 h of storage at 5°C (Ijaz and Ducharme, 1995) though, the viability and motility decline after 18 hours (Parlevliet *et al.* 1992).

Honey consists of ≈200 useful components and important sugar (da Silva *et al.* 2016). It has potent antibacterial and antioxidant properties (Zoheir *et al.* 2015) as well as free radicals scavenging activity (Chua *et al.* 2013). Manuka honey, monofloral honey derived from the manuka tree (*Leptospermum scoparium*), contains flavonoids, phenolic acids, and other compounds (Wang *et al.* 2024, Cianciosi *et al.* 2023) which account for its immunomodulatory, antimicrobial, antioxidative and wound healing properties (Opsivac *et al.* 2023). Therefore, present study was undertaken to investigate the effect of Manuka honey supplemented in Kenney (milk-based) and Dimitropoulos (egg-based) extenders on the spermatozoa characteristics and biochemical parameters of chilled stallion semen.

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MATERIALS AND METHODS

Animals and semen collection: The experimental procedures were approved by the Animal Welfare and Ethics Committee of Faculty of Veterinary Medicine, Benha University, Egypt with an ethical approval number (BUFVTM 02-02–23). Semen collection was conducted at the Armed Forces Equestrian Club, Cairo, Egypt, and the laboratory work was conducted at Armed Forces Veterinary Hospital, Cairo, Egypt during August-October 2021.

Four Arabian stallions of proven fertility, body condition scores 4-5 (scale of 1= poor to 9= extremely fat), 4-8 years old, 350-400 kg body weight were used in the study. Stallions were kept individually in 5 × 3 m stalls and were fed barseem (4 kg/head) or drees (1 kg/head) and concentrates mix 9% protein (6 kg/head). Mineral salts and water were available *ad libitum*.

Semen samples (n= 48 ejaculates) were collected, twice weekly, for six consecutive weeks on an estrus mare using a pre-warmed Missouri artificial vagina (Nasco, Ft. Atkinson, WI, USA) according to Arifiantini *et al.* (2006).

Extenders preparations, semen extension and evaluation: Milk-based (Kenney) and Egg-based (Dimitropoulos) extenders used for semen dilution were prepared according to Kenney *et al.* (1975) and Arifiantinia *et al.* (2013), respectively.

The Kenney (Ken) extender was prepared from Non-fat dry skimmed milk 2.40 g, glucose 4.90 g, $NaHCO_3$ (8.4%) 2.00 ml and Milli-Q water 100 ml. Dimitropoulos (DM) extender composed of 30%-part A (glucose 2.00 g and fructose 2.00 g in Milli-Q water 100 ml), 50%-part B (sodium citrate dihydrate 2.00 g, glycine 0.94 g and sulfanilamide 0.35 g in Milli-Q water 100 ml), and 20% of egg yolk. The prepared DM mixed extender was centrifuged at 1200 ×g for 20 min, and the supernatant was used as the extender. Antibiotic mixture (1000 IU/ml Penicillin and 1 mg/ml Streptomycin) was added to each extender before use. Each extender (Ken and DM) was divided into aliquots and manuka honey (manuka honey +20 UMF/850 MGO - 250g, Mānuka Health®, New Zealand, purchased from CAFLEX, Egypt) was added at 0 % (control), 1, 2, and 4 % (V/V) per each at room temperature with good mixing (Kandiel and Elkhawagah, 2017).

The gel-free semen samples with minimal > 100×10^6 spermatozoa/ml, and motility and livability > 70% were passed for semen extension (Rečková *et al.* 2022) to a final concentration 25×10^6 sperm/ml (i.e. 1:4 dilution rate). Extended semen was gradually cooled to 4 °C within two hour (El-Sheshtawy *et al.* 2016) and kept in refrigerator for 72 hours.

For evaluation of the changes in semen characteristics during storage, semen aliquots (0.5 ml), were warmed at 35 °C for 10 min at 12-hour intervals before evaluation for progressive motility, livability, and morphology (Sheshtawy *et al.* 2016; Łącka *et al.* 2016; El- Dowsett *et al.* 1984, respectively). Spermatozoa progressive motility was examined using a pre-warmed stage of a phase contrast microscope (×40). The sperm viability and total abnormalities were assessed using eosin (5%) and nigrosine (10%) stains.

For assessment of sperm-free biochemical parameters, semen samples were centrifuged at 1800 ×g (Hermal Compact Centrifuge Z206-A, Germany) for 5 min (Hoogewijs *et al.* 2010), and the supernatant was used for evaluation of acid phosphatase (Catalog # AC1011, Randox, Spain) and aspartate aminotransferase (Catalog # AS3804, Randox, Spain) spectrophotometrically (Jenway 6300, USA). The BNPNA method was used to assess acrosin activity (acrosin amidase activity µIU/ml) according to Cui *et al.* (2000). Catalase (CAT), superoxide dismutase (SOD), and Glutathione peroxidase (GPX) activities as well as malondialdehyde (MDA) concentration were determined using appropriate commercial kits (Catalog #K773-100, #K335-100, #K762-100, and # K739, respectively, BioVision, USA).

Statistical analysis: The data were analyzed for normality with Shapiro-Wilk test and presented as mean ± SE. One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was achieved using

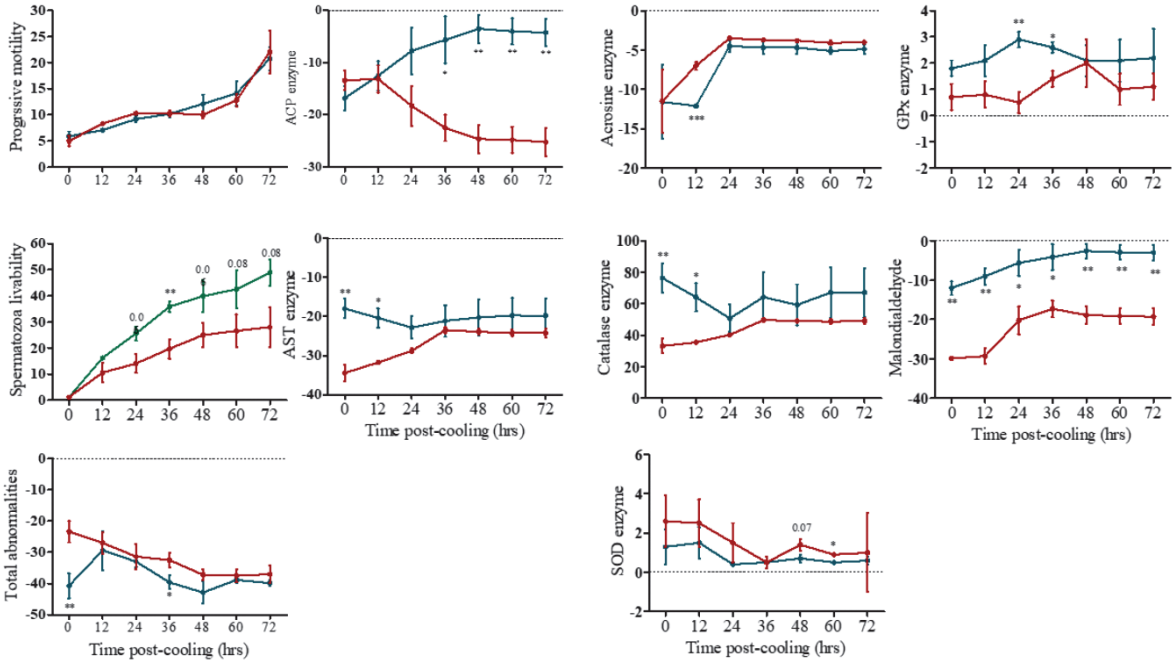


Fig.1. Comparative effect of Kenney (-●-) and Dimitropoulos (-●-) extenders with supplementation of Manuka honey 2% on the relative changes (%) in stallion chilled characters. *, ** and *** demarked significant changes at P< 0.05, 0.01 and 0.001, respectively, independent *t*-test. ACP, AST, SOD, GPx referred to acid phosphatase, aspartate aminotransferase, Superoxide dismutase, and glutathione peroxidase, respectively.

SPSS (Ver. 25) to compare between different concentrations of manuka honey in each extender. The best concentration of Manuka honey in Kenney and Dimitropoulos extenders was compared using an independent student *t*-test based on the relative change to their controls. The statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Storage of semen at low temperature (cooling/freezing) adversely affects stallion sperm motility, viability, and fertility (Aurich, 2005; Elkhawagah *et al.* 2020 & 2024). Stallion spermatozoa during cold storage have declining energy sources and is susceptible to cold shock and lipid peroxidation (Chow *et al.* 1986; Gautier and Aurich, 2022). In the current study, manuka honey strengthened Kenney and Dimitropoulos extenders in preserving stallion chilled semen through reduced cell-free (released) enzymes, enhanced catalase activity, and reduced lipid peroxidation. Spermatozoa livability and total abnormalities, acrosine and catalase relative changes were superior with manuka honey 2% in Kenney. Meanwhile ACP, AST, and MDA relative changes were better with MH 2% in Dimitropoulos extenders (Fig. 1).

In the present study, 2% of manuka honey in DM extender improved ($P< 0.05$) sperm motility at 72 hours of cold-storage periods. In the meantime, manuka honey (2%) in Kenney extender markedly improved spermatozoa livability at 24 -72 hrs post-cooling. These results presumed the value of manuka honey to skimmed milk extender in superimposing sperm livability, and egg yolk

Table 1. Effect of Manuka honey supplementation on acid phosphatase enzyme activity (U/L) in chilled equine extended semen

Extender	Time (hr)	Control	MH 1%	MH 2%	MH 4%	P value
Kenney extender	0	24.97±0.75^{a}	24.70±0.81^{a}	20.73±0.32^{b}	26.03±1.11^{a}	0.007
	12	25.97±0.77^{a}	25.80±0.79^{a}	22.70±0.61^{b}	26.63±0.93^{a}	0.03
	24	27.03±0.67^{ab}	26.90±0.70^{ab}	24.93±1.03^{b}	27.77±0.55^{a}	0.08
	36	28.17±0.38	27.90±0.32	26.63±1.58	28.73±0.35	ns
	48	28.60±0.78	28.47±0.81	27.63±1.47	31.03±1.18	ns
	60	28.43±0.81^{ab}	28.33±0.72^{ab}	27.33±1.41^{b}	31.93±1.33^{a}	0.08
	72	28.07±0.84	27.90±0.84	26.93±1.45	29.30±0.50	ns
Dimitropoulos extender	0	27.00±0.72^{b}	26.93±0.76^{b}	23.37±0.44^{b}	31.93±1.83^{a}	0.004
	12	27.97±0.81^{b}	27.80±0.91^{b}	24.27±0.55^{c}	32.83±0.41^{a}	0.001
	24	30.47±1.56^{ab}	30.23±1.59^{ab}	24.80±0.61^{b}	32.87±2.40^{a}	0.005
	36	32.60±1.37^{b}	32.53±1.32^{b}	25.20±0.61^{c}	36.80±1.02^{a}	0.001
	48	33.50±1.46^{a}	33.33±1.47^{a}	25.20±0.45^{b}	37.53±1.58^{a}	0.001
	60	33.27±1.42^{a}	33.30±1.37^{a}	24.97±0.52^{b}	37.17±1.90^{a}	0.002
	72	33.00±1.42^{a}	32.90±1.46^{a}	24.63±0.58^{b}	35.67±1.60^{a}	0.002

Values with different superscript letters in the same row were significantly different

Table 2. Effect of Manuka honey supplementation on aspartate aminotransferase enzyme activity (U/L) in chilled equine extended semen

Extender	Time (hr)	Control	MH 1%	MH 2%	MH 4%	P value
Kenney extender	0	187.47±3.55^{a}	155.67±1.50^{b}	153.60±1.80^{b}	160.10±1.62^{b}	0.001
	12	194.87±4.32^{a}	156.37±1.41^{bc}	154.93±1.47^{c}	164.33±2.44^{b}	0.001
	24	205.80±4.51^{a}	160.17±2.52^{c}	158.70±2.65^{c}	174.70±0.87^{b}	0.001
	36	199.43±6.01^{a}	157.63±3.20^{c}	156.83±3.38^{c}	173.03±0.54^{b}	0.001
	48	196.33±7.32^{a}	157.13±3.18^{c}	156.03±3.30^{c}	172.37±0.83^{b}	0.001
	60	194.23±6.87^{a}	156.00±3.70^{c}	155.30±3.24^{c}	170.47±0.90^{b}	0.001
	72	193.90±6.79^{a}	155.47±3.77^{b}	154.83±3.24^{b}	168.60±0.31^{b}	0.001
Dimitropoulos extender	0	197.27±0.93^{a}	130.33±4.41^{b}	129.40±4.12^{b}	135.70±5.02^{b}	0.001
	12	201.60±0.23^{a}	139.07±2.11^{b}	137.70±1.23^{b}	142.93±4.79^{b}	0.001
	24	203.13±0.35^{a}	144.93±1.54^{b}	144.63±1.54^{b}	150.67±3.82^{b}	0.001
	36	204.57±0.30^{a}	156.83±2.05^{b}	156.47±1.96^{b}	162.53±3.62^{b}	0.001
	48	204.33±0.32^{a}	155.77±1.94^{b}	155.53±1.94^{b}	161.17±3.76^{b}	0.001
	60	203.97±0.27^{a}	155.07±1.79^{b}	154.60±1.71^{b}	160.63±3.53^{b}	0.001
	72	203.43±0.34^{a}	154.57±1.89^{b}	154.23±1.91^{b}	160.17±3.78^{b}	0.001

Values with different superscript letters in the same row were significantly different

Table 3. Effect of Manuka honey supplementation on acrosin enzyme activity (µU/L) in chilled equine extended semen

Extender	Time (hr)	Control	MH 1%	MH 2%	MH 4%	P value
Kenney extender	0	350.67±8.19^{a}	320.00±7.64^{b}	309.33±12.13^{b}	335.00±5.03^{ab}	0.043
	12	299.00±6.08^{a}	277.00±10.02^{ab}	262.67±4.63^{b}	287.33±6.36^{a}	0.034
	24	263.67±2.60^{a}	251.33±2.40^{b}	251.67±1.76^{b}	259.67±4.10^{ab}	0.038
	36	261.67±2.60^{a}	249.67±2.33^{b}	249.33±1.45^{b}	258.00±4.16^{ab}	0.034
	48	259.67±2.60^{a}	248.33±2.03^{b}	247.33±1.45^{b}	256.00±4.16^{ab}	0.035
	60	257.00±2.65^{a}	246.67±1.76^{b}	244.00±2.08^{b}	253.67±4.48^{ab}	0.046
	72	253.33±2.91^{a}	244.67±1.76^{ab}	241.00±1.53^{b}	250.33±5.24^{ab}	0.097
Dimitropoulos extender	0	352.33±6.49	319.00±12.42	311.33±10.84	327.67±11.89	0.11
	12	283.33±9.53	269.67±7.88	263.33±8.41	279.00±9.64	0.43
	24	263.67±2.91	258.33±3.18	254.33±2.60	260.33±3.18	0.24
	36	261.67±2.91	256.33±3.18	252.00±2.65	257.67±3.76	0.26
	48	260.00±2.89	254.67±2.91	250.00±2.65	255.33±3.48	0.21
	60	258.00±2.89	252.67±2.91	247.33±2.03	253.00±3.46	0.15
	72	255.67±2.60	250.67±2.91	245.33±2.03	250.67±3.48	0.15

Values with different superscript letters in the same row were significantly different

Table 4. Effect of Manuka honey supplementation on catalase enzyme activity (U/L) in chilled equine extended semen

Extender	Time (hr)	Control	MH 1%	MH 2%	MH 4%	P value
Kenney extender	0	87.47±3.55^{b}	155.67±1.50^{a}	153.60±1.80^{a}	160.10±1.62^{a}	0.001
	12	94.87±4.32^{c}	156.37±1.4^{1a}b	154.93±1.47^{b}	164.33±2.44^{a}	0.001
	24	105.80±4.51^{c}	160.17±2.52^{b}	158.70±2.65^{b}	174.70±0.87^{a}	0.001
	36	96.33±7.32^{c}	157.13±3.18^{b}	156.03±3.30^{b}	172.37±0.83^{a}	0.001
	48	99.43±6.01^{c}	157.63±3.20^{b}	156.83±3.38^{b}	173.03±0.54^{a}	0.001
	60	94.23±6.87^{c}	156.00±3.70^{b}	155.30±3.24^{b}	170.47±0.90^{a}	0.001
	72	93.90±6.79^{c}	155.47±3.77^{a}	154.83±3.24^{a}	168.60±0.31^{a}	0.001
Dimitropoulos extender	0	97.27±0.93^{b}	130.33±4.41^{a}	129.40±4.12^{a}	135.70±5.02^{a}	0.001
	12	101.60±0.23^{b}	139.07±2.11^{a}	137.70±1.23^{a}	142.93±4.79^{a}	0.001
	24	103.13±0.35^{b}	144.93±1.54^{a}	144.63±1.54^{a}	150.67±3.82^{a}	0.001
	36	104.57±0.30^{b}	156.83±2.05^{a}	156.47±1.96^{a}	162.53±3.62^{a}	0.001
	48	104.33±0.32^{b}	155.77±1.94^{a}	155.53±1.94^{a}	161.17±3.76^{a}	0.001
	60	103.97±0.27^{b}	155.07±1.79^{a}	154.60±1.71^{a}	160.63±3.53^{a}	0.001
	72	103.43±0.34^{b}	154.57±1.89^{a}	154.23±1.91^{a}	160.17±3.78^{a}	0.001

Values with different superscript letters at the same row were significantly different

to keep sperm motility after long storage. The positive effect of manuka honey on sperm motility and livability might be attributed to its content of phenolic compounds (anti-oxidants) and supplemented monosaccharides (~ 61% of the sugar content) high in fructose/glucose content (Aboagla and Terada, 2004). The natural components of honey serve as a source of energy and cold shock protector in semen extenders and improve the sperm motility and livability during chilling (Kandiel and Elkhawagah, 2017). In addition to its contents of sugars, natural honey contains vitamins and amino acids that improve the sperm's livability (El-Sheshtawy *et al.* 2016). Former studies affirmed the improving effect of honey on the extended chilled semen of stallions (El-Sheshtawy *et al.* 2016), bucks (Olayemi *et al.* 2011), bulls (El-Sheshtawy *et al.* 2014), and buffalo bulls (Kandiel and Elkhawagah, 2017).

Semen storage at 4-5 ºC exposes spermatozoa to cold shock which damages sperm acrosomal and plasma membranes due to ROS production (Ball, 2008; Arifiantinia *et al.* 2013; Loureiro *et al.* 2020). The supplementation of Ken and DM extender with manuka honey markedly reduced the released ACP at a concentration of 2 % (Table 1) and AST at different concentrations (1-4%) during storage (Table 2). Besides, MH 1 and 2 % in Ken extender decreased the release of sperm acrosin (Table 3). This indicated the sperm membrane-protection potential of manuka honey. AST is the main enzyme in ATP production by mitochondria (Colenbrander *et al.* 1992). Therefore, damage in the sperm midpiece leads to release of AST to the seminal plasma, which disturbs the ATP production and sperm motility. The phenolic contents of honey have been reported to protect the sperm cell against

Table 5. Effect of Manuka honey supplementation on malondialdehyde level (nmol/mg) in chilled equine extended semen

Extender	Time (hr)	Control	MH 1%	MH 2%	MH 4%	P value
Kenney extender	0	34.97±0.75^{a}	34.70±0.81^{a}	30.73±0.32^{b}	36.03±1.11^{a}	0.007
	12	35.97±0.77^{a}	35.80±0.79^{a}	32.70±0.61^{b}	36.63±0.93^{a}	0.03
	24	37.03±0.67^{ab}	36.90±0.70^{ab}	34.93±1.03^{b}	37.77±0.55^{a}	0.128
	36	38.17±0.38	37.90±0.32	36.63±1.58	38.73±0.35	0.405
	48	38.60±0.78	38.47±0.81	37.63±1.47	37.70±2.15	0.942
	60	38.43±0.81	38.33±0.72	37.33±1.41	35.27±2.19	0.408
	72	38.07±0.84	37.90±0.84	36.93±1.45	39.30±0.50	0.44
Dimitropoulos extender	0	47.00±0.72^{a}	46.93±0.76^{a}	32.97±0.65^{b}	45.27±1.53^{a}	0.001
	12	47.97±0.81^{a}	47.80±0.91^{a}	33.91±0.86^{c}	42.83±0.41^{b}	0.001
	24	43.80±2.24^{a}	43.57±2.21^{a}	34.80±0.61^{b}	46.20±1.15^{a}	0.001
	36	42.60±1.37^{a}	42.53±1.32^{a}	35.20±0.61^{b}	46.80±1.02^{a}	0.001
	48	43.50±1.46^{a}	43.33±1.47^{a}	35.20±0.45^{b}	47.53±1.58^{a}	0.001
	60	43.27±1.42^{a}	43.30±1.37^{a}	34.97±0.52^{b}	43.83±2.23^{a}	0.01
	72	43.00±1.42^{a}	42.90±1.46^{a}	34.63±0.58^{b}	45.67±1.60^{a}	0.002

Values with different superscript letters in the same row were significantly different

temperature alteration oxidative stress through its electron- or hydrogen-liberation capacity by neutralizing reactive oxidants (Beretta *et al.* 2005; Moskaug *et al.* 2005).

Several antioxidants including catalase, SOD and glutathione enzymes have been detected in stallion seminal plasma, preserving sperm metabolism by decreasing lipid peroxidation induced by ROS (Baumber *et al.* 2003). In this study, supplementation of Kenny and DM extenders with different concentrations of honey (1-4%) enhanced CAT activities and decreased the level of MDA (2% honey) in cold storage (Tables 4 and 5). It has been previously reported that Manuka honey is a potent antioxidant and antibacterial (Aljady *et al.* 2000; Zoheir *et al.* 2015). In addition, honey flavonoids contain free radical scavenging activity, which inhibits lipid peroxidation (Chua *et al.* 2013).

In conclusion, manuka honey worked as a potent bio-additive that substantially optimized stallion chilled semen. MH incorporation at 2 % level yielded distinctive, extender-specific benefits. It strengthened semen characteristics and antioxidant defense when formulated with Kenney extender. It maximized membrane integrity and minimized lipid peroxidation when paired with the Dimitropoulos extender.

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