



Comparative study of chicken egg yolk and quail egg yolk in two chilled canine semen extenders

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Abstract

The aim of this work was to substitute chicken egg yolk with quail egg yolk in two semen extenders and to evaluate the quality of the extended canine semen following chilled storage. Semen was pooled from male dogs ($n=4$) of about 18-months old and body weight of about 28 kg. Four extenders were tested: (1) tris buffered chicken egg yolk extender (2) tris buffered quail egg yolk extender, (3) skimmed milk chicken egg yolk extender and (4) skimmed milk quail egg yolk extender. Semen was diluted with corresponding extender in the ratio 1:4. The diluted semen samples were analyzed for motility, mass activity, viability, abnormalities percentage and pH for three consecutive days. There was no significant difference ($P>0.05$) between chicken egg yolk and quail egg yolk in either tris diluent or skimmed milk extender with respect to pH, mass activity and sperm motility. Samples stored in both the tris and skimmed milk-based extenders with quail egg yolk displayed greater viability than those in chicken egg yolk but the difference was not significant ($P>0.05$). Viability, mass activity and sperm motility decreased as treatment days increased in both chicken and quail egg yolk extenders. Results showed that a pH of 6.5 was maintained from day 0 to day 3. There was no difference in semen quality between chicken and quail egg yolk in either the tris diluent or skimmed milk extender ($P>0.05$). It was recommended that quail egg yolk could be substituted for chicken egg yolk in the two canine semen extenders. Further modifications of the diluents with quail egg yolk might produce an improved result.

Keywords: Canine, Chicken Chilled, Egg yolk, Extenders, Quail, Semen

Introduction

Semen collection and storage enhances the distribution of valuable genetic material and is an economical and safe alternative compared to whole

animal transport. However, global canine semen trade is relatively limited in most developing countries due to high cost of commercial diluents

(Diaz *et al.*, 2013). In these areas, dogs are transported to long distances for mating and the pedigree is not guaranteed (Diaz *et al.*, 2013). Cheaper, easy to use, efficient and safe diluents are needed before semen preservation can be more widespread and accessible. As against frozen-thawed semen, chilled canine semen can be deposited in the vagina with high fertility rate (Linde-Forsberg, 1991). It is easier to handle and ship chilled semen compared to frozen semen (Diaz *et al.*, 2013).

It is a simple practice to chill semen, whereas, processing of frozen semen requires costly equipment and trained personnel that are not always available. The fertility rate of artificial insemination (AI) is normally greater for chilled semen than frozen semen under standard conditions (Linde-Forsberg, 1995). Considering these facts, cooling appears to be the logical initial approach to canine semen preservation. Additionally, semen could be pre-cooled and transported to semen banks for freezing and permanent storage (Linde-Forsberg, 1991).

Cooling involves a number of factors that may damage the sperm plasma membrane (Linde-Forsberg, 1995). However, canine semen can be diluted in extenders, chilled and stored at 4°C for many days (Linde-Forsberg & Forsberg, 1993). Extenders shield spermatozoa, gives room for motility and fertility preservation over time by stabilizing plasma membrane, providing energy substrates, buffers, antibiotic activity and osmotic balance over time (Linde-Forsberg, 1995). Milk is a common component of semen extenders in many species, with high success rate both *in vitro* and *in vivo* (Maxwell & Salamon, 1993; Batellier *et al.*, 2001). Skim milk proteins buffer semen pH and may also chelate any heavy metal ions (Jones & Martin, 1973; Maxwell & Salamon, 1993; Batellier *et al.*, 2001). The inclusion of egg yolk to skim milk extender enhances the survival of spermatozoa during chilled storage (Salamon & Maxwell, 2000) because its phospholipid fraction provides protection to sperm and acrosomal membranes against cold shock (Jones & Martin, 1973; White, 1993). It has also been postulated that milk and egg yolk have a combined, protective effect on sperm because of their uniform action on the sequestration of seminal plasma proteins (Iguer-ouada & Verstegen, 2001). Binder sperm proteins are seminal plasma proteins which are detrimental to sperm cells. Understanding this fact, skim milk with or without egg yolk could be a cheap, practical diluent for cooling canine semen. Reports on skim milk as a diluent for cooling canine semen with acceptable *in vitro* results are rare (Rota *et al.*, 1995). Skim milk with

egg yolk has shown post-thaw semen quality similar to those observed with a tris-based buffer in dogs (Rota *et al.*, 2001). Egg yolk is believed to improve stored semen quality but its advantage over skim milk has not been described. Tris buffers are more stable at high temperature and other different environmental conditions than bicarbonate and sodium citrate buffers (Gadea, 2003). Tris-citric acid-egg-yolk-fructose based extenders (known as the Uppsala diluents) are most widely applied extenders for the preservation of canine sperm (Linde-Forsberg, 2002). During chilling, the second sperm-rich fraction can be diluted with tris-citric acid-fructose diluent with 20% egg yolk in a proportion of 1:3 to 1:4. The diluted semen can be preserved at 4-5°C for upwards of 10 days as determined by the type of extender used and the initial semen quality (Iguer-ouada & Verstegen, 2001). Today, most practitioners use Tris-buffer as an extender for canine semen preservation (Iguer-Ouada & Verstegen, 2001). Tris preserves sperm energy by reducing fructolysis (Rodrigues, 1997).

Quail eggs have a unique biochemical composition and are superior to other avian species nutritionally. Quail eggs are rich in fat- and water-soluble vitamins, proteins, amino acids, macro and microelements (Tunsaringkarn *et al.*, 2012). The eggs have low cholesterol, triglyceride and saturated fatty acids (Bayomy *et al.*, 2017). They are endowed with antioxidants, minerals and vitamins while, albumen composition is fairly constant (Robert, 1977). Chicken and quail eggs are not significantly different in the cholesterol level of their yolk (Bragagnalo & Rodriguez-Amaya, 2003; Tunsaringkarn *et al.*, 2012). However, some research results documented higher cholesterol content in quail egg yolk than chicken egg yolk (Kaźmierska *et al.*, 2005). Use of chicken and quail egg yolks in semen diluents have been compared in Poitou jackass where results showed that after the freeze-thaw process, quail egg yolk enhanced the percentages of motile and progressively undulating spermatozoa compared to chicken egg yolk (Trimeche *et al.*, 1997). The chemical composition of chicken and quail egg yolks are related, but quail egg yolk have significantly higher phosphatidylcholine, less phosphatidylethanolamine and a lower proportion of polyunsaturated to saturated fatty acids than chicken egg yolk (Tunsaringkarn *et al.*, 2012). The enhancement of motility for frozen-thawed Poitou jackass spermatozoa using frozen-thawed quail egg yolk compared to chicken egg yolk may be related to the variations in constituents of the two yolks (Trimeche

et al., 1997). Egg yolk from different breeds of poultry has shown significant differences in their potential to shield or maintain motility of chilled spermatozoa of West African Dwarf (WAD) bucks. The variations were assigned to different biochemical constituents of the egg yolks (Daramola *et al.*, 2013). Considering the desire to develop improved diluents for chilled canine semen this work was designed to modify the two major diluents used in dogs by substituting chicken egg yolk with quail egg yolk in their preparations and evaluating the quality of the extended semen stored under chilled condition.

Materials and Methods

Experimental design

This work focused on two canine semen extenders; (Tris buffered canine semen extender and skimmed milk extender). Both stored under chilled condition at 5°C were prepared. The experiment involved four groups of extenders via (1) tris buffered chicken egg yolk extender, (2) tris buffered quail egg yolk extender (3) skimmed milk chicken egg yolk extender and (4) skimmed milk quail egg yolk extender. The four groups were diluted at the dilution ratio of 1:4 with canine semen and chilled to 5°C. Each of the four diluted samples were aliquoted into 8 properly labeled sterile containers. The experiments were observed each day for the period of 3 days and mass activity score (0-5), motility, live and abnormality percentages and pH were recorded. The experiments involved taking out a sample each day, warm and observing under microscope to assess the mentioned parameters.

Experimental animals

The animal experiment followed the principles of the Laboratory animal care (Canadian Council on Animal Care Guide, 1993). Semen was pooled from four mature dogs of an average age of 18 months and body weight of 28 kg; based on history of proven fertility, recommendation by a breeder and thorough breeding soundness examination. The dogs were selected from Kubwa, Abuja.

Semen collection

Semen was collected from the dogs by manual massage. The dogs were restrained by the use of a mouth guard and the owner holding the collar. The collector had a graduated tube fitted with a nylon cone on his left hand, while he gradually exposed the penis from the prepuce with his right hand in a massaging motion. As soon as erection occurred the nylon cone with the collecting tube was fixed tight to

the penis with the nylon holding the bulbo glandis tight over a period of 20 mins until ejaculation occurred. About 14 ml of semen from each dog was collected into a graduated tube and maintained at 37°C in a water bath as described by Althouse *et al.* (1991). Only samples that were at least milky in color and with motility of 80% and above were pooled.

Pre dilution examination

Volume of semen was observed and recorded; color was graded from creamy, milky to watery.

Microscopic examination

A drop of fresh semen was made on a prewarmed glass slide on a warm stage and covered with a warm cover slip. The sample was viewed under the microscope starting from X 4 objective magnification to observe collective directional movement of spermatozoa (wave motion) and then to X 10 objective magnification to observe individual motility.

Macroscopic examination

As described by Sirivaidyapong *et al.* (2001), a drop of fresh semen was made on a prewarmed glass slide and a drop of warm eosin-nigrosin stain was made on the drop of semen. A thin smear was made on the glass slide and preserved until analysis. The smear was used to assess percent viability and morphology. The slides were viewed at x100 objective lens and 100 cells were counted per slide. Live cells repelled the vital stain because of the integrity of the nuclear membrane. Dead cells appeared pink because of loss of integrity of the nuclear membrane. Also, head abnormalities, mid piece and tail abnormalities were assessed by one of the authors.

Preparation of semen extenders

Preparation of the buffer

Tris buffer was prepared as described by Silva *et al.* (2000): In brief, 2.9 g tris, 1.7 g citric acid and 1.25 g fructose was added into 20 ml of distilled water in a flat bottom flask. The mixture was shaken together until the salt dissolved completely. The solution was then made up to 100 ml by adding distilled water.

Harvesting of egg yolk

Freshly laid eggs were collected from quail and poultry farms. These eggs were cleaned and disinfected using 70 % alcohol. They were cracked carefully into two, such that the albumen drained off from the crack until little of it was left with the yolk. The yolk was then carefully dropped on Whatman

filter paper which absorbed what was left of the albumen. The yolks were collected into beakers.

Extender preparation

The tris diluent was made by pouring 80 ml of tris buffer in 100 ml graduated cylinder and making up to 100 ml with 20 ml of egg yolk (quail or chicken) and addition of Penicillin/Streptomycin. 0.6 ml (100,000 i/u and 200 mg respectively).

Skimmed milk diluent was prepared based on producer’s instruction: 475 g of dried skimmed milk (Marvel®) was weighed and added into 500 ml of distilled water. Then, 80 ml of prepared skimmed milk was measured in a 100 ml measuring cylinder and the solution was made up to 100 ml with egg yolk (chicken or quail) Penicillin/Streptomycin. 0.6 ml (100,000 i/u and 200 mg respectively) were added.

Semen dilution and chilling

Dog semen was diluted 1:4 by taking 1 ml of fresh semen into 4 ml of four extenders (previously described) at 37°C.

Semen was chilled by using ice blocks in a 50ml beaker. Ten milliliters each of diluted semen samples were transferred into a 10 ml sample tube each, these were tightly covered and dipped into the beaker of ice blocks and were allowed to chill for 1 h. The four diluents were gently rocked and dispensed into tubes of 5 ml capacity. 0.5 ml of extended semen was dispensed into test tubes. Comprising (1) 8 tubes for tris chicken egg yolk extender (2) 8 tubes for tris quail egg yolk extender (3) 8 tubes for skimmed milk quail egg yolk extender and (4) 8 tubes for skimmed milk chicken egg yolk extender. The tubes were labeled properly and were kept in Ice park before transfer to a fridge at 4°C for further chilling and storage.

Extended semen in the four groups of extenders was accessed starting from day zero post dilution to day 3

post dilution for pH, mass activity, motility and percentage viability and morphology.

Statistical analyses of data

Data were expressed as means and standard error of mean (SEM). Data were analyzed using descriptive statistics and ANOVA with SPSS/PC computer program (Version 20.0, SPSS®, Chicago IL, USA). Differences with confidence values of P < 0.05 were considered statistically significant (Daniel, 1991).

Results

Results showed that the pH of the four groups remained the same from day 0 to day 3 of the experiment; pH of 6.5 was maintained. There was no significant difference in semen characteristics when semen was stored in chicken egg yolk and quail egg yolk in both the tris diluent or skimmed milk extender (p>0.05). Sperm morphology in diluted semen was not significantly different (p>0.05) between semen extended in chicken and quail egg yolks in either milk or tris diluents (Table 1). There was no significant difference (p>0.05) in mass activity of semen between chicken and quail egg yolks in both skimmed milk and tris diluents. However, mass activity reduced as observation days increased (p>0.05). Mass activity stopped on day 2 for milk diluents for both chicken and quail egg yolks while mass activity for tris diluent continued to day 3 for both chicken and quail egg yolks (Figures 1 and 2). The same trend was observed for sperm motility (Figures 3 and 4). Live sperm percentage showed that the milk diluents sustained live cells up to day 3 just as the tris diluents did (Figures 5 and 6). Live percentage showed that quail egg yolk had more life cells than chicken egg yolk in both tris and skimmed milk diluents but the difference was not significant (p>0.05). Live percentage decreased as treatment days increased.

Table 1: Mean and standard deviation of percentage abnormalities post dilution of dog semen in both skimmed milk and tris diluents using chicken and quail egg yolk in each diluent.

Days	Sk Milk + Quail	Sk Milk + Chicken	Tris + Quail	Tris + Chicken
0	2.00 ± 0.00	1.00 ± 0.00	4.00 ± 0.00	2.00 ± 0.00
1	5.00 ± 2.28	3.00 ± 4.56	0.00 ± 0.00	4.00 ± 2.28
2	3.00 ± 2.28	2.00 ± 0.00	0.00 ± 0.00	6.00 ± 2.82
3	1.00 ± 0.00	6.00 ± 5.64	2.00 ± 0.00	1.00 ± 0.00

There was no significant difference in the percentage abnormalities between chicken and quail egg yolks (p>0.05).

Discussion

According to the results the four groups of diluents maintained a pH of 6.5 for the period of the study. This is an indication that the four diluents had equal buffering capacity. Milk diluents are effective buffers. The buffering action may be due to its protein composition which includes casein and whey. These two proteins can serve as protein buffers. This observation is consistent with the report of Cunha (2002), which stated that milk has a buffering capacity, bactericidal action and an adequate viscosity for the maintenance of spermatozoa in a liquid medium (Tatyane *et al.*, 2015). Quail egg yolk did not make any difference in the stability of the milk based canine semen diluent. Similarly, the tris diluent is an effective buffer and is a well-known buffer used in many biological studies especially in molecular biology. The observation agrees with the report that tris is extensively used in biochemistry and molecular biology as a component of buffer solutions such as in TAE (buffer solution containing a mixture of Tris base, acetic acid and EDTA) and TBE buffers, especially for solutions of nucleic acids (Tris - Wikipedia, 2018). Biological buffers like tris are important because they can maintain a stable pH despite influences that might otherwise shift the pH. Tris is a commonly used buffer in biological labs (Tris - Wikipedia, 2018). Quail egg yolk did not differ from the

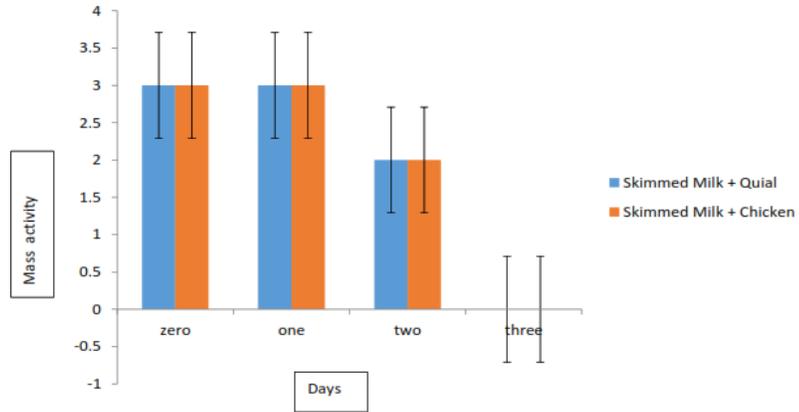


Figure 1: Daily mass activity post dilution of dog semen in skimmed milk extenders using chicken and quail egg yolks independently. There was no significant difference in the mass activity between chicken and quail egg yolks ($p>0.05$)

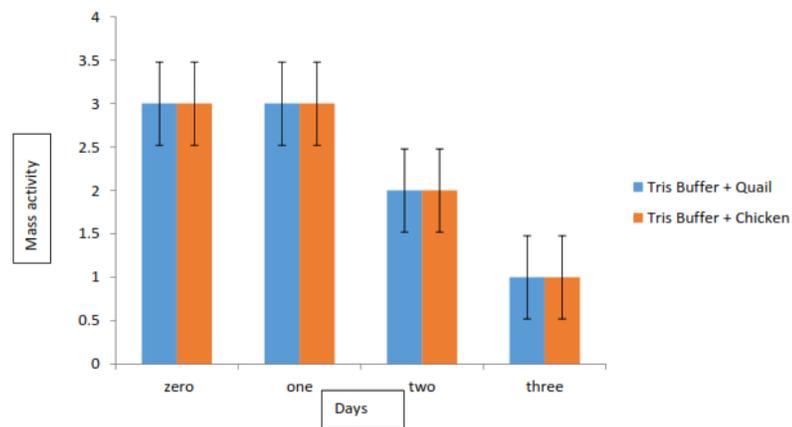


Figure 2: Daily mass activity post dilution of dog semen in Tris buffer extenders using chicken and quail egg yolks independently. There was no significant difference in the mass activity between chicken and quail egg yolks ($p>0.05$)

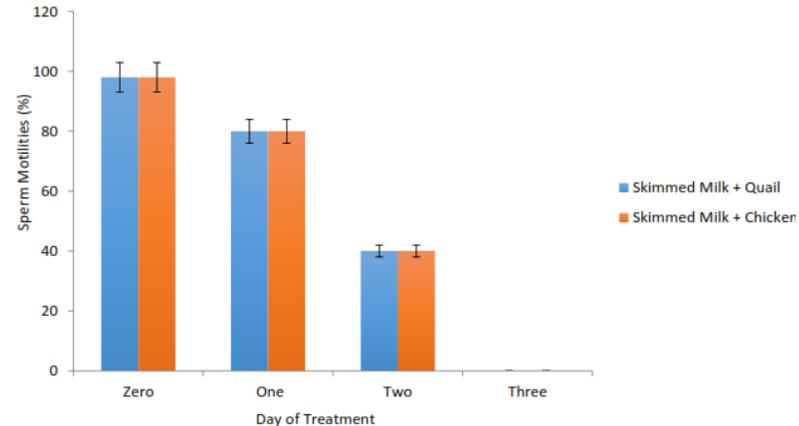


Figure 3: Daily sperm motility post dilution of dog semen in skimmed milk extenders using chicken and quail egg yolks independently. There was no significant difference in the sperm motility between chicken and quail egg yolks ($p>0.05$)

chicken egg yolk in maintaining pH of the diluents. Concerning the mass activity and sperm motility, skimmed milk diluent lost motility within 2 days for both chicken and quail egg yolks addition whereas, tris diluent maintained motility till day 3. This observation is consistent with the report that under standard operations semen diluted in milk is difficult to evaluate (Iguer-ouada & Versteegen 2001). The optical clarity of milk extender is poor because of the fat droplets. Light passing through the sample is reflected and refracted making the observation of individual sperm difficult (Michael, 2018). It also supports the document that stated that the addition of sodium citrate in yolk phosphate buffered extender improved sperm survival to three days at 5°C and sperm visibility was also improved by dissolving the fat globules (Gadea, 2003). Motility assessment in milk diluents is difficult due to poor optical clarity. Milk is a viscous fluid and this contributes to poor vision through the microscopic slides. Viscosity of milk has also been confirmed by Tatyane *et al.* (2015) who reported that milk has an adequate viscosity for the maintenance of spermatozoa in a liquid medium.

The live percentage proved that the milk diluent was as good as the tris diluent because live cells were

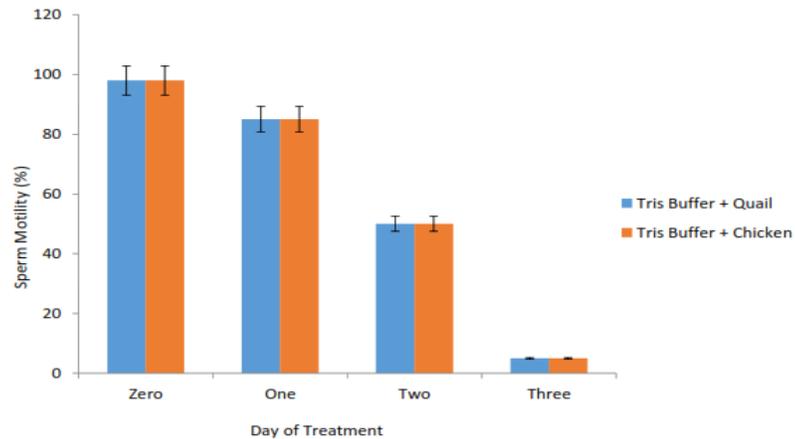


Figure 4: Daily sperm motility post dilution of dog semen in tris buffer extenders using chicken and quail egg yolks independently. There was no significant difference in the sperm motility between chicken and quail egg yolks ($p>0.05$)

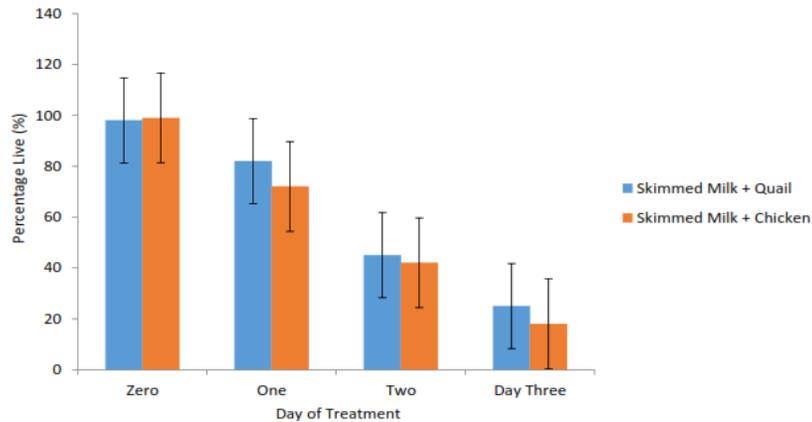


Figure 5: Daily percentage lives post dilution of dog semen in skimmed milk extenders using chicken and quail egg yolks independently. There was no significant difference in the percentage live between chicken and quail egg yolks ($p>0.05$)

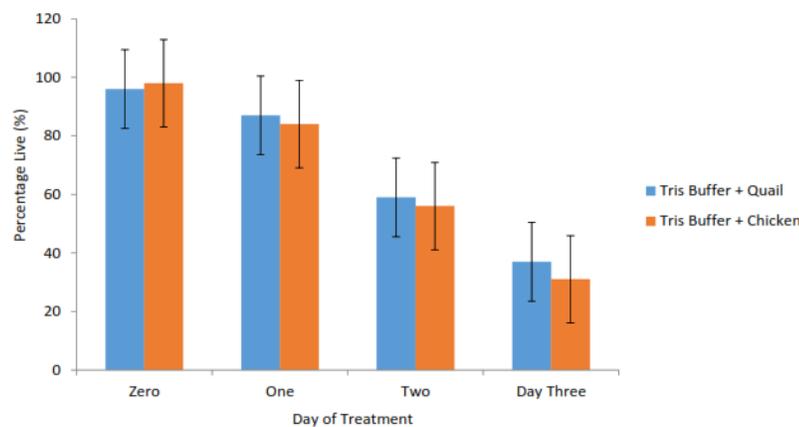


Figure 6: Daily percentage live post dilution of dog semen in tris buffer extenders using chicken and quail egg yolks independently. There was no significant difference in the percentage live between chicken and quail egg yolks ($p>0.05$)

present up to day 3 just as it appeared in the tris diluents. Reviewing the live percentage, it would be good to observe that quail egg yolk has a better potential in maintaining canine semen in the two diluents tested although the differences were not significant. Quail egg yolk showed more live percentage compared to chicken egg yolk in all the days of observation covering tris diluent and skimmed milk diluent. This also supports the work of Daramola *et al.* (2013) who reported that quail egg yolk improved the percentages of motile and progressively undulating spermatozoa and the movement characteristics compared with chicken egg yolk in Poitou jackass. This effect may be due the chemical compositions of the two egg yolks. Quail egg yolk contains more phosphatidylcholine and saturated fatty acids. These factors may be lending support to the sperm plasma membrane which is made of lipoproteins. Sperm survival after ejaculation depends largely on the interaction of its plasma membrane with binder sperm proteins present in the seminal plasma. In general the storage time of 3 days in the present study may be consistent with milk extenders as used in simple veterinary clinics, but the tris diluent did not fall in line with other reporters that stated that semen diluted in tris iluent can be preserved at 4-5°C for upwards of 10 days as determined by the type of extender used and the initial semen quality (Iguer-ouada & Verstegen, 2001). This variation from our work may be due to tris EDTA used or due to non-separation of the sperm rich fraction of the ejaculate from the seminal plasma before dilution.

It is concluded that quail egg yolk may substitute chicken egg yolk in tris and milk canine semen extenders as there was no significant difference between the two egg yolks in supporting the semen parameters evaluation.

Further modifications of the diluents with quail egg yolk may yield an improved canine diluent.

Conflicts of Interest

The authors declare no conflict of interest.

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