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Viability assessment of spermatozoa in large falcons (*Falco* spp.) using various staining protocols

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Abstract

Viability assessment is an important part of semen analysis, and various live/dead staining protocols have been used in semen of avian species. Results of live/dead count differed between dyes, staining protocols and bird species, impeding comparability between studies and requiring species-specific comparisons of viability stains. In raptor semen, similar comparisons are absent. Thus, the aim of the present study was to compare eight conventional viability stains. Eosin blue 2% [EB], eosin blue 2% with the addition of 3% sodium citrate [EB2], eosin blue–nigrosin 5% [EBN5], eosin yellow–nigrosin 5% [EYN5], eosin yellow–nigrosin 10% [EYN10], eosin blue–aniline blue [EBA], eosin yellow–aniline blue [EYA] and bromophenol blue–nigrosin [BBN] were evaluated in comparison with the fluorescence stain SYBR® Green–propidium iodide [SYBR-PI] in spermatozoa of falcons. The comparison was performed using conventional light microscopy which is applicable in breeding centres, veterinary practices and field studies. Additionally, live/dead stains were correlated to motility values of the same samples to validate sperm viability. Light microscopy using EB and using SYBR-PI enabled an effective and clear differentiation between alive and dead spermatozoa of falcons. Motility values correlated significantly and strongly with EB only ($r = .629$; $p < .001$), but not with any other stain used in the study. Therefore, our results suggest EB as the most suitable stain for viability assessment in the semen of large falcons.

KEYWORDS

andrology, assisted reproduction, live/dead count, semen analysis, sperm vitality, supravital stain

1 | INTRODUCTION

Sperm viability assessment is an important part of a standardized semen analysis in veterinary and human reproductive medicine (Björndahl et al., 2004; WHO, 2010). For this purpose, dye exclusion methods are established since the 1940s (Lasley, Easley, & McKenzie, 1942), offering a differentiation between intact, alive, but (temporarily) immobile spermatozoa and defect (dead) spermatozoa. This differentiation is important for the prediction of fertility and the estimation of semen quality, especially in semen samples where the amount of motile spermatozoa is low (Björndahl, Söderlund, & Kvist, 2003). Live/dead stains are able to penetrate the defect

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cellular membrane and to colour intracellular structures of the spermatozoa. In contrast, the membrane of vital spermatozoa is impermeable and the cell remains unstained.

In contrast to standardized protocols in human andrology (WHO, 2010), common guidelines are lacking in veterinary medicine and especially in avian medicine. However, various stains and numerous staining protocols have been used for semen analysis in birds. Conventional live/dead stains, such as eosin blue [EB] (Behncke, 2002; Fischer, Neumann, Wehrend, & Lierz, 2014; Lierz, Reinschmidt, Müller, Wink, & Neumann, 2013; Schneider et al., 2017, 2018; Stelzer, Crosta, Bürkle, & Krautwald-Junghanns, 2005; Stelzer, Schmidt, Sobiraj, & Krautwald-Junghanns, 2009), eosin–nigrosin [EBN, EYN] (Bailey, 2002; Blanco, Long, Gee, Donoghue, & Wildt, 2008; Chalah & Brillard, 1998; Hartley, Dawson, Lindsay, McCormick, & Wishart, 1999; Madeddu et al., 2009; Saint Jalme, Lecoq, Seigneurin, Blesbois, & Plouzeau, 2003), eosin blue–aniline [EBA] (Bailey, 2002; Varga, Barna, & Almási, 2003) and bromophenol blue–nigrosin [BBN] (Kamar, 1959; Wilson, Warnick, & Gutierrez, 1969), have been used beside several fluorescent probes, such as SYBR® 14/Green–propidium iodide [SYBR-PI] and SYBR® 14/Green–ethidium homodimer 1 [SYBR-EthD-1] (Donoghue, Garner, Donoghue, & Johnson, 1995; Garner & Johnson, 1995; Garner, Johnson, Yue, Roth, & Haugland, 1994; Garner, Pinkel, Johnson, & Pace, 1986; Klimowicz-Bodys, Batkowski, Ochrem, & Savič, 2012). The latter, dual colorimetric fluorescent methods allow the coloration of intracellular structures of cells with damaged outer cellular membranes in one colour and of the genome of vital spermatozoa in a different colour (Chalah & Brillard, 1998).

The usage of several protocols and stains limits comparability between different studies, even if spermatozoa of the same bird species are examined. In this regard, studies in semen of roosters demonstrated a large variability of sperm viability results by using different live/dead staining protocols, underlining the need of standardized procedures (Wilson et al., 1969). Nevertheless, it is difficult to decide which results of which live/dead staining protocol are best suited to demonstrate true sperm viability.

To some degree, sperm viability may be validated by sperm motility assessment, because both parameters of semen analysis are reported to correlate well (Klimowicz-Bodys et al., 2012). Correlations of different viability stains and motility values in birds have only been studied in semen of chickens, turkeys and pigeons (Chalah & Brillard, 1998; Donoghue et al., 1995; Klimowicz-Bodys et al., 2012). In this regard, high positive correlations between EYN and SYBR-PI and the proportions of motile spermatozoa (MOT) and of progressively motile spermatozoa (PMOT) have been demonstrated in pigeon semen by using a computer-assisted semen analysis (Klimowicz-Bodys et al., 2012). There has been no significant difference between eosin–nigrosin and SYBR-PI demonstrated in fresh semen, but viability results of the fluorescence stain decreased after storing semen samples for 6 and 24 hr (Klimowicz-Bodys et al., 2012). The study in rooster semen demonstrated that SYBR-PI is better suited for viability assessment in fresh and cryopreserved semen than eosin–nigrosin, with a higher percentage of mortality in stored semen post-freezing and post-thawing (Chalah & Brillard, 1998). Likewise, in turkey semen the combination of SYBR with PI has been demonstrated as one of two most effective live/dead stains of the various stains tested in the panel of different dilutions (Donoghue et al., 1995; Friars & Chatterjee, 1969). In semen of cockatiels, SYBR-PI enabled an efficient viability assessment as well as the conventional stain eosin blue 2% (Schneider et al., 2017, 2018), but detailed correlations to sperm motility have not been investigated in this species.

In birds of prey, comparative studies of live/dead stains are lacking. For viability assessment in semen samples of raptors EBN/EYN (Bailey, 2002; Blanco et al., 2008; Hartley, 1998; Madeddu et al., 2009), EB (Fischer, Garcia de la Fuente, Wehrend, & Lierz, 2011), aniline blue eosin (Bailey, 2002), SYBR-PI (Blanco, Gee, Wildt, & Donoghue, 2002) and SYBR-EthD-1 (Bailey, 2002; Bailey, Holt, Bennett, Barton, & Fox, 2008) have been used. These studies revealed a limited usability of EBN and aniline blue eosin in semen of falcons, as differentiation between alive and dead spermatozoa seemed to be difficult or even impossible in some samples (Bailey, 2002; Bailey et al., 2008; Gee, Morrell, Franson, & Pattee, 1993; Hoolihan & Burnham, 1985). In macroscopically uncontaminated semen samples of eagles and large falcons, sperm viability has been demonstrated higher compared to samples with urate contaminations using eosin–nigrosin, even though further information is absent in this study and no comparison to other stains was performed (Blanco et al., 2002). In raptor semen, pleomorphic cells (Villaverde-Morcillo et al., 2017) and round bodies (Bailey et al., 2008; Hartley, 1998) complicate semen evaluation and in most studies it is not clear whether spermatozoa, spermatids and round bodies were distinguished or counted in combination during viability assessment.

The aim of the first part of the present study was to compare viability assessment by using eight different conventional live/dead staining protocols in semen of large falcons as the first study of that kind in a raptor species. In the second part of the study, the best conventional live/dead staining protocol was compared to the dual fluorescent stain combination SYBR-PI. Furthermore, all viability results using different live/dead stains were correlated to motility values of spermatozoa within the same sample according to previous studies (Klimowicz-Bodys et al., 2012).

2 | MATERIALS AND METHODS

2.1 | Animals and semen collection

Eighteen, 4 – 19 years old, male falcons (gyrfalcons, *Falco rusticolus*, $n = 7$; peregrine falcons, *Falco peregrinus*, $n = 4$; hybrid falcons, *Falco rusticolus X Falco cherrug*, $n = 7$) were kept in outdoor aviaries with access to natural light in a commercial breeding centre for large falcons. The birds were kept and imprinted to humans according to standard procedures (Boyd, Boyd, & Dobler, 1977; Temple, 1972). All birds served as semen donors and had already

fathered offspring prior to the study. Water was provided ad libitum and food (rats, quail, 1-day chickens) was offered twice daily by the animal keeper to maintain bonding to the imprinted birds. Semen collections were performed by the animal keeper using the established abdominal massage method with a 48- to 72-hr interval (Bird & Lague, 1976; Samour, 2004) according to management plans and schedules of the breeding centre. Aliquots of routine semen samples, which were not needed for artificial insemination, were examined according to availability, dispensing the need for separate/additional semen collection for scientific purposes. Handling of the animals and semen collection was done in accordance with species conservation (CITES) and animal welfare laws (Spanish Policy for Animal Protection RD53/2013), and all procedures in the breeding centre were approved and controlled by the competent authority (Executive Council of Catalonia, Barcelona, Spain; NIF-B25493008).

2.2 | Motility assessment, staining preparation and viability assessment

2.2.1 | Motility assessment and comparison of eight conventional live/dead stains

In the first part of the study, viability results using eight conventional live/dead stains (Figure 1) were compared and correlated to sperm motility in 25 semen samples of 13 falcons. To this end, all semen samples were diluted to equal parts (1:2) with 6-Hour SemAid (PHL Associates Inc., Davis, CA, USA) prior to motility and to viability analysis.

Firstly, 5 μl of the diluted semen sample was placed on a prewarmed slide (37°C), covered with a coverslip and used to assess sperm motility (MOT) and progressive sperm motility (PMOT) according to standard procedures (Fischer, Neumann, Wehrend, et al., 2014) under a light microscope at 400-fold magnification (Hund H500; Helmut Hund GmbH, Wetzlar, Germany). Briefly, sperm cells (spermatozoa and spermatids, excluding round bodies) in five fields of view, beginning in the centre, followed by clockwise examination of four additional fields, were screened systematically.

With the intention of covering a wide range of sperm viability and sperm motility, especially to cover also low viability and motility values, besides immediate examination, semen analysis was also performed at delayed points in time after semen collection and dilution (>2.5–52 hr; $\bar{x} \pm SD$: 6.76 ± 11.8 hr; median: 26 hr). As the semen sample volume was limited, it was only sufficient for one viability evaluation using all of the following eight conventional staining protocols. Thus, comparative assessment was performed only at one time point per semen sample.

As live/dead stains, eosin blue 2% [EB], eosin blue 2% with the addition of 3% sodium citrate [EB2], eosin blue–nigrosin 5% [EBN5], eosin yellow–nigrosin 5% [EYN5], eosin yellow–nigrosin 10% [EYN10], eosin blue–aniline blue [EBA], eosin yellow–aniline blue [EYA] and bromophenol blue–nigrosin [BBN] were evaluated in comparison with the same semen sample. To this end, staining solution and diluted semen sample were mixed by five times resuspension, incubated for 30 s on a slide and smeared in the manner of making a blood smear (WHO, 2010) before air drying at room temperature. All viability smears were evaluated in a light microscope at 400-fold magnification, starting at the middle third of the smear and moving in a meandering pattern towards the beginning of the smear. Per smear, during the same assessment, firstly 200 cells (spermatozoa and spermatids) and secondly 200 spermatozoa were counted as dead (stained) or alive (unstained) using a digital cell counter (Assistent® Counter AC-8, Glaswarenfabrik Karl Hecht GmbH & Co. KG, Sondheim vor der Rhön, Germany). Spermatids inside round bodies and other non-sperm shaped, pleomorphic cells were not counted. Two viability values were given per smear—the percentage of viable cells with spermatozoa and spermatids in a mix (total viability—TOV) and the percentage of viable spermatozoa (only spermatozoa viability—OSV).

EB was used as 2% standard mixture according to studies in psittacines (Neumann, Kaleta, & Lierz, 2013; Stelzer et al., 2005).

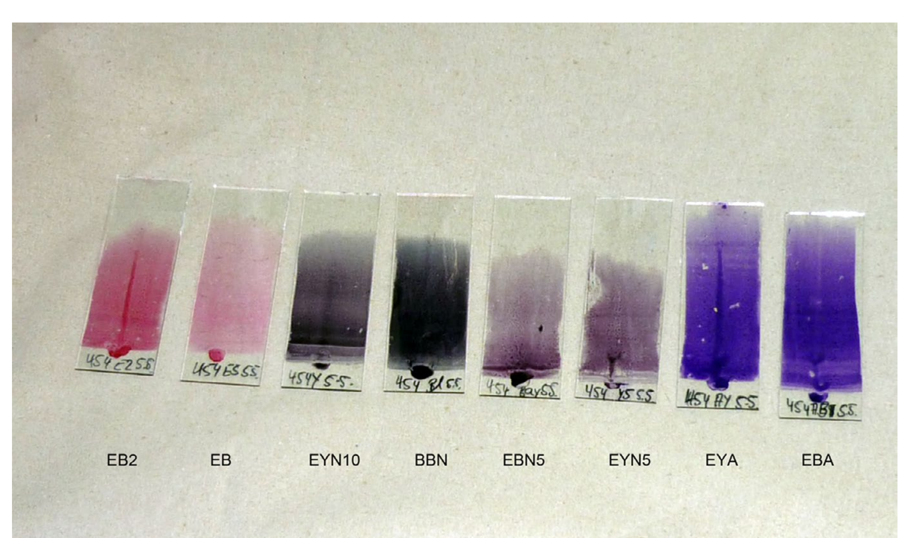

FIGURE 1 Stained semen smears for sperm viability assessment using different live/dead stains in semen samples of large falcons. From left to right: EB2 (eosin blue 2% plus 3% sodium citrate), EB (eosin blue 2%), EYN10 (eosin yellow with 10% nigrosin), BBN (bromophenol blue–nigrosin), EBN5 (eosin blue with 5% nigrosin), EYN5 (eosin yellow with 5% nigrosin), EYA (eosin yellow aniline) and EBA (eosin blue–aniline)

Additionally, 3 g tri-sodium citrate hydrate (Merck KGaA) was added to this mixture and examined as EB2 in comparison (Behncke, 2002). Eosin yellow was mixed with 5% nigrosin [EYN5] (Dott & Foster, 1972) and 10% nigrosin [EYN10] (Carothers & Beatty, 1975; Hancock, 1951), respectively. Likewise, eosin blue was mixed with 5% nigrosin [EBN5] as described previously (Bailey, 2002). All three eosin–nigrosin stains were used according to studies in griffon vultures (*Gyps fulvus*) (Madeddu et al., 2009).

Eosin blue–aniline stain [EBA] was prepared according to a previous study in New Zealand falcons (*Falco novaeseelandiae*) (Bailey, 2002). Additionally, eosin yellow was used in the same combination with aniline blue, using the same protocol but eosin yellow instead of eosin blue [EAY].

Bromophenol blue–nigrosin [BBN] was prepared and used according to a study in bull spermatozoa (Schnabel, 2009).

Particular information to all stains and mixtures, including staining properties, was depicted in Table 1.

2.2.2 | Motility assessment and comparison of EB and SYBR-PI

The second part of the study was performed in a facility with the possibility to perform fluorescence microscopy to compare the best conventional live/dead stain with the dual fluorescent dye combination SYBR-PI. To this end, 33 semen samples of five falcons were used; semen dilution and motility assessment were done in the same way as described above.

Fluorescence stain SYBR-PI was used according to previous studies (Bailey, 2002; Blanco et al., 2002; Fischer, Neumann, Wehrend, et al., 2014). Briefly, 5 μl diluted semen sample was incubated with 2.5 μl pre-mixed SYBR-PI as per the manufacturer's instructions (Minitube, Tiefenbach, Germany) for ten minutes at room temperature in a light-protected tube. Afterwards, the 7.5 μl stained semen sample was transferred in standardized counting chamber (Leja® Standard Count 4 Chamber, Leja Products B.V., Nieuw-Vennep, The Netherlands) and TOV and OSF were assessed immediately, using a fluorescence light source (Olympus U-RFL-T, Olympus Corporation Tokyo) and a fluorescence microscope (Olympus BX41 TF). SYBR labels DNA in living cells with green fluorescence (excitation: 488 nm; emission: 516 nm; Garner et al., 1994) and PI colours cell nuclei red (excitation: 530 nm; emission: 617 nm; Donoghue et al., 1995; Garner & Johnson, 1995; Garner et al., 1986).

Viability assessment using the conventional and the fluorescent stains were started in alternating order to avoid time-dependent differences of viability assessment. Results of both viability assessments were correlated with each other and with sperm motility values afterwards.

3 | STATISTICAL EVALUATION

Correlation analysis of sperm viability and sperm motility was performed using the Pearson correlation coefficient and the program GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

A comparison of the means of all eight conventional viability stains in the first part of the study via one-way analysis of variance (ANOVA) with repeated measures (program BMDP2V) followed by the pairwise comparison with the Student–Newman–Keuls test (SNK test) for dependent samples and a pairwise correlation analysis including bivariate scatter plots were done using the program package BMDP Statistical Software, release 8.1 (Dixon, 1993). Moreover, a regression analysis regarding the possible influence of the duration between semen collection and analysis was added (program BMDP6D). Potential differences between the live/dead stains depending on the amount of TOV and OSF within the samples were identified via Bland–Altman analysis (Bland & Altman, 1986; Dixon, 1993).

The comparison between the conventional and the fluorescent stain in the second part of the study was examined via one-factorial ANOVA with repeated measures. Means were compared via paired test for dependent samples using the SNK procedure. Moreover, a pairwise correlation analysis and Bland–Altman analysis was performed as done for the eight conventional viability stains previously.

4 | RESULTS

4.1 | Microscopic evaluation

Results of the microscopic examination are depicted in Table 1. Visual differentiation between intact and damaged spermatozoa and spermatids was effectively possible using EB (Figure 2a) and EB2 (Figure 2e). Comparing both stains, EB2 seemed to be coloured darker red compared with EB. Therefore, in some areas of the stained semen smears the differentiation of dead cells from the background was more difficult using EB2 compared with EB stain. Spermatids, spermatozoa and round bodies were assessable in both stains.

Overlay with the dark background stain using EBN5 exacerbated the identification of dead spermatozoa and required to perform live/dead counts in bright areas of the smear only. The red to pink coloration of dead sperm cells was weak and difficult to see (Figure 2c). In comparison with EB and EB2, a fewer number of cells was visible in the semen smear of the same sample. Spermatids were often covered by the background stain, while round bodies were visible as bright, round areas only.

Viability stain EYN5 appeared to be similar to EBN5. However, dead sperm were only partly stained red, but partly coloured wine red and thus difficult to differentiate from the background.

Using EYN10, spermatozoa seemed to have a dark, distinct margin and were clearly visible in front of the dark background stain, even though narrow and small sperm structures (e.g. flagella or acrosome) were difficult to identify. Dead cells consisted mainly of swollen, vaguely delimited, pleomorphic spermatids, which had a slightly

TABLE 1 Stain composition, smear preparation, staining properties and microscopic results of various conventional live/dead stains in semen samples of large falcons

Stain/abbreviation/stain composition	Smear preparation/intended colour/stain properties	Microscopic results in semen samples of large falcons	Staining references
Eosin blue 2% [EB] *2 g eosin blue*[a]*, 100 ml dist. water*	2 µl stain solution + 2 µl diluted semen LS: unstained DS: red	LS: unstained; DS: red (light red-pink to dark red); RB: red; Remark: Good differentiation between LS and DS; bright pink background, clear identification of sperm/spermatid structures (mid-piece, flagella, acrosome) and of RB	Neumann et al., 2013, Stelzer, 2004 Stelzer et al., 2005
Eosin blue 2% plus tri-sodium citrate [EB2] *2 g eosin blue*[a]*, 3 g tri-sodium citrate hydrate*[a]*, 100 ml dist. water*	2 µl stain solution + 2 µl diluted semen LS: unstained DS: red	LS: unstained; DS: red (red-pink to dark red); RB: red; Remark: Differentiation of LS and DF more difficult compared with EB due to darker background colour (dark pink to red), clear identification of sperm/spermatid structure (flagella, acrosome and mid-piece) and of RB; identification in dark parts of the smear impaired	Behncke, 2002
Eosin blue 5% nigrosin [EBN5] *1 g eosin blue*[b]*, 2 g nigrosin*[c]*, 100ml dist. water*	4 µl stain solution + 2 µl diluted semen LS: unstained with a dark background contrast through nigrosin DS: pink	LS: unstained, but often overlay with background stain DS: weak red to pink (using EBN partly wine red); RB: visible as bright, round areas Remark: Exacerbated identification of DS, requiring to perform live/dead counts in bright areas of the smear, due to overlay with the dark background stain; spermatids often swollen, vaguely delimited, pleomorphic cells with a slightly and barely visible reddish colour; using EYN10 spermatozoa seemed to have a dark, distinct margin, increasing visibility; compared with EB a lower number of spermatids and spermatozoa was visible, due to covering-over by the background stain; narrow and small structures (e.g. flagella or acrosome) difficult to identify	Bailey, 2002
Eosin yellow 5% nigrosin [EYN5] *0,67 g eosin yellow (Gurr)*[c]*, 5 g nigrosin*[c]*, 100 ml dist. water*			Dott & Foster, 1972
Eosin yellow 10% nigrosin [EYN10] *5 g water soluble eosin yellow (Gurr)*[c]*, 300 ml 10% nigrosin*			Carothers & Beatty, 1975, Hancock, 1951
Eosin blue–aniline [EBA] *0.80 g aniline blue, water soluble (cotton blue)*[a]*, 0.20 g eosin blue*[b]*, 10 ml 92G solution*[d]	5 µl stain solution + 2 µl diluted semen LS: unstained with a dark purple contrast through aniline DS: pink	LS: clear white in contrast to background DS: weakly red; RB: bright and homogenous pink; Remark: LS contrasts well with the dark purple (EBA) or pink (EYA) background stain; DS very difficult to identify in contrast to the background; high number of staining artefacts; exacerbated identification of spermatids and small and narrow sperm structures (e.g. flagella, mid-piece and acrosome)	Bailey, 2002
Eosin yellow–aniline [EYA] *as above but eosin y*[c]			Bailey, 2002
Bromophenol blue–nigrosine [BBN] *2 g bromophenol blue*[a]*, 10 g nigrosine, 100 ml dist. water*	4 µl stain solution + 2 µl diluted semen LS: unstained with a dark contrast through nigrosin DS: blue	LS: white to light blue with a clear dark margin; DS: stained slightly darker in bluish grey and thus difficult to differentiate from the background stain; RB: weakly stained in bluish grey Remark: differentiation of spermatozoa and spermatids from the background stain is very difficult, especially identification of DS is difficult due to weak staining and covering-over by the background stain	Schnabel, 2009

Abbreviations: dist., distilled; DS, dead/defect spermatozoa/spermatids; LS, living spermatozoa/spermatids; RB, round bodies.

[a]Merck KGaA, Darmstadt, Germany.

[b]Minitube, Tiefenbach, Germany.

[c]VWR International GmbH, Langenfeld, Germany.

[d]92G solution: 1.92 g sodium glutamate (monohydrate) 0.103 M, 0.08 g magnesium acetate (tetrahydrate) 0.004 M; 0.13 g tripotassium citrate (monohydrate) 0.004 M; 0.062 g sodium acetate (anhydrous) 0.51 M; 0.6 g glucose 0.033 M, 100 ml distilled water.

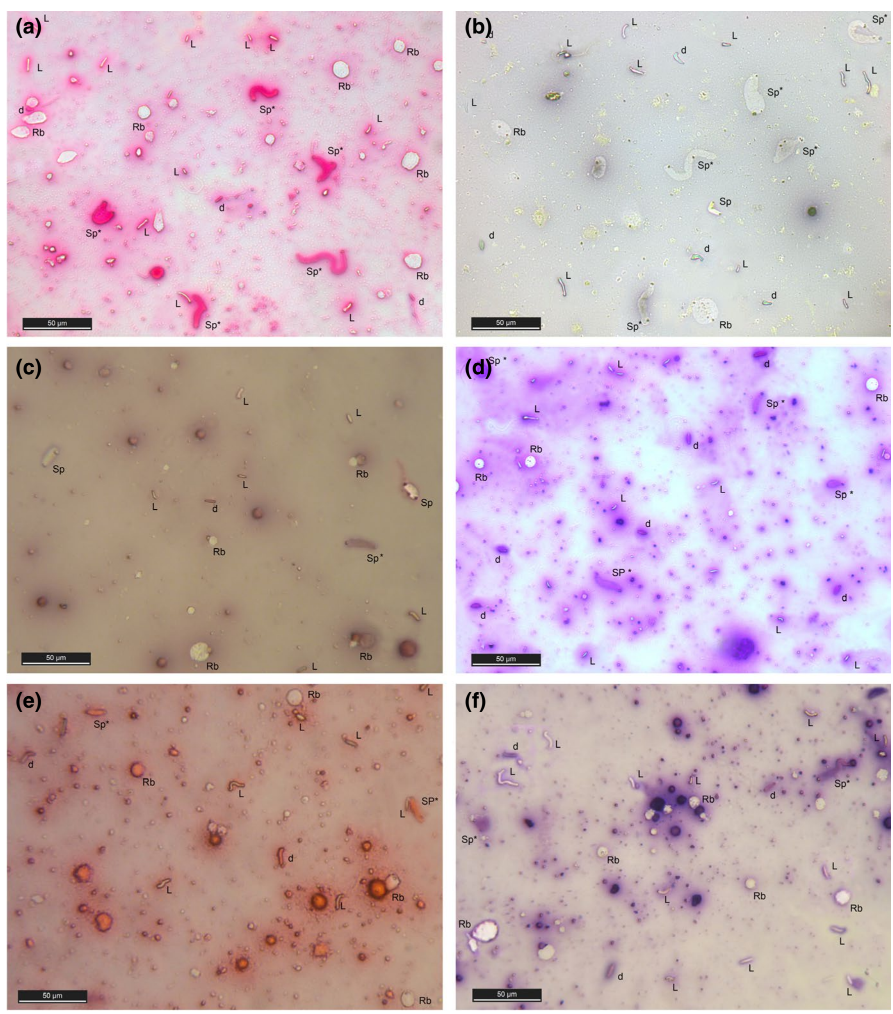

FIGURE 2 Viability assessment using different live/dead stains in semen samples of large falcons. Using the staining protocols, living spermatozoa (L) and viable spermatids (Sp) remain unstained; dead spermatozoa (d) and dead spermatids (Sp*) are stained specifically according to the used protocol. Round bodies (Rb) are variable in staining properties, partially stained and partially unstained. Live/dead stains: (a) Eosin B 2% (EB): dead spermatozoa and spermatids are stained red; (b) bromophenol blue–nigrosin (BBN): dead spermatozoa are stained blue and dead spermatids bluish grey; (c) eosin blue with 5% nigrosin (EBN5): dead spermatozoa and spermatids are stained pink; (d) eosin blue–aniline (EBA): dead spermatozoa and spermatids are stained pink/purple. Note the high number of staining artefacts; (e) eosin B 2% plus sodium citrate 3% (EB2): dead spermatozoa and spermatids are stained red; (f) eosin yellow–aniline (EYA): dead spermatozoa and spermatids are stained purple. Note the high number of staining artefacts

and barely visible reddish colour compared with the background. The number of visible spermatids and round bodies using EYN10 was low compared with EB and EB2.

In EBA (Figure 2d) and EYA (Figure 2f), spermatozoa contrasted well with the dark purple (EBA) or pink (EYA) background stain, respectively. Alive spermatozoa were visible in clear white in contrast to this background stains. However, dead spermatozoa were stained weakly red and thus were very difficult to identify in contrast to the background. Round bodies appeared bright and homogenously pink. A high number of staining artefacts and dark coloured areas complicated the identification of spermatids and small and narrow sperm structures such as sperm tail and acrosome. There seemed to be no differences between EBA and EYA.

Using BBN (Figure 2b), alive spermatozoa appeared white to light blue with a clear dark margin. Dead spermatozoa were stained slightly darker in bluish grey and were thus difficult to differentiate from the background stain. Likewise, round bodies and spermatids were weakly stained in bluish grey and therefore also difficult to identify.

Using SYBR-PI live/dead stain in semen samples, living spermatozoa and viable spermatids were stained green and dead spermatozoa and spermatids appeared red (Figure 3). Thus, a differentiation was easily possible. Round bodies were variable in staining properties, partially stained green and partially stained red.

4.2 | Statistical results

The comparison of eight conventional live/dead stains with sperm motility (MOT, PMOT) in the first part of the study revealed only for EB a significant, but very weak correlation of MOT with OSV (r = .406, p = .038). All other conventional live/dead stains did not show significant correlations with MOT and PMOT.

Likewise, in the second part of the study for EB OSV correlated significantly, but stronger with MOT (r = .629, p < .001) and PMOT (r = .510, p = .002). The fluorescence dye SYBR-PI showed a significant correlation with MOT (r = 0.370, p = .034) and PMOT (r = .284, p = .055) as well, but the correlations were very weak with a Pearson coefficient below 0.5 only. Results of correlation analysis are depicted in Table 2.

Comparing sperm viability results, EB correlated significantly with EB2 (r = .723; p < .001), EYN5 (r = .653; p = .015), and EBN (r = .479; p = .033). No significant correlation was present with EYN10, EBA, EYA, and BBN.

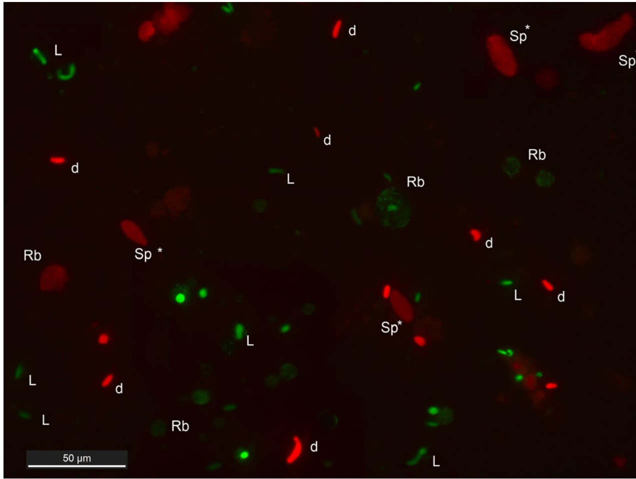

FIGURE 3 Viability assessment using SYBR® Green–propidium iodide live/dead stain in semen samples of large falcons. Living spermatozoa (L) and viable spermatids (Sp) are stained green, and dead spermatozoa (d) and dead spermatids (Sp*) are stained red. Round bodies (Rb) are variable in staining properties, partially stained green and partially stained red

TABLE 2 Correlation of the live/dead stains eosin B (EB) and SYBR Green–propidium iodide (SYBR-PI) with sperm motility in semen samples of large falcons using Pearson correlation coefficient

	EB			SYBR-PI	
(n = 33)		r	p-value	r	p-value
TOV	MOT	**.541**	**.001**	–	–
	PMOT	**.439**	**.011**	–	–
OSV	MOT	**.629**	**<.001**	**.370**	**.017**
	PMOT	**.510**	**.002**	.284	.055

Abbreviations: EB, eosin blue; MOT, total sperm motility; n, number of samples; OSV, only spermatozoa viability; PMOT, progressive sperm motility; SYBR-PI, SYBR Green–propidium iodide; TOV, total sperm cell viability, spermatozoa and spermatids counted together.

Bold values represent statistically significant correlations.

Global ANOVA analysis of variance and covariance with repeated measures revealed that means of all stains differed significantly from EB (p < .01: EYN5, EY10, EBA, EYA, BBN; p < .05: EB). Pairwise comparison of means of the various stains confirmed significant differences of the various stains from EB, as depicted in Table 3.

In this regard, Bland–Altman analysis revealed that these differences between EB and EBN, EYN5, EBA and EYA depended on the amount of viable spermatozoa, increasing with a decreasing number of living sperm cells. Therefore, differences are negligible if viability is high.

Means of SYBR-PI differed significantly from EB as well in global ANOVA analysis (p < .01) and pairwise comparison ($\bar{x} \pm SD$: 5.5 ± 15.5%, p = .048), but Bland–Altman analysis failed to prove a significant dependence on the amount of the viability level (OSV/TOV). All results of pairwise correlation analysis, t test and Bland-Altman analysis are depicted in Table 3.

5 | DISCUSSION

Sperm viability assessment has been shown as an important part of semen analysis (Björndahl et al., 2003) with conventional manual and automated methods being established in avian medicine. In this regard, the sperm chromatin dispersion assay and flow cytometry have been reported as objective tools to assess avian sperm DNA fragmentation and sperm viability (Fernández, Cajigal, López-Fernández, & Gosálvez, 2011; Johnston et al., 2020; Mercies, Chenier, Plante, & Buhr, 2000). Although such modern and automated methods have been demonstrated as very effective, accurate and fast tools (Chalah & Brillard, 1998; Donoghue et al., 1995; Klimowicz-Bodys et al., 2012), they are mostly not available in general practice, breeding centres or field studies. Thus, manual microscopic sperm viability methods are still important and widely used for sperm viability analysis. For this reason, we intended the evaluation of conventional live/dead stains in the first part of the study and the comparison with a modern fluorescence dye in the second part of the study; both backed up by a correlation with sperm motility results of the same samples. In particular, eosin blue, a single colorimetric stain that has been successfully used in sharp-tailed grouse (Schneider et al., 2019) and various psittacine species (Behncke, 2002; Fischer, Neumann, Purchase, et al., 2014; Lierz et al., 2013; Schneider et al., 2017, 2018; Stelzer et al., 2005, 2009) was evaluated in semen samples of falcons for the first time. Furthermore, eosin blue was compared in two different preparations (EB and EB2) to various established staining combinations of eosin yellow and eosin blue with different proportions of the background stains nigrosin or aniline (EYN5, EBN5 EYN10, EYA, EBA), respectively, to bromophenol blue–nigrosin (BBN) and the fluorescence stain SYBR® Green–propidium iodide (SYBR-PI).

As the quality of staining depends on several factors, such as content of ionizable salts, osmotic pressure, pH, stain concentration, time of staining, and time of drying (Emik & Sidwell, 1947; Mayer, Dale Squires, Bogart, & Oloufa, 1951), all samples were treated identically and in accordance to previous studies. Nevertheless, a comparison to previous studies and reproducibility are limited due to the lack of precise information about dye preparation and composition and the staining protocol in many studies. However, this is crucial, because methods of staining may be very variable even if the same dye is used (Björndahl et al., 2004). For example, eosin–nigrosin has been used in various concentrations of eosin and nigrosin and in one- and two-step methods. On the one hand in roosters, semen and eosin were mixed before adding nigrosin by placing one drop of diluted semen beside one drop of eosin yellow and three drops of nigrosin on a slide (Lake & Stewart, 1978). In semen of vultures (Madeddu et al., 2009), one drop of semen was mixed with five drops of eosin yellow–nigrosin dye according to methods in porcine medicine (Pintado, de La Fuente, & Roldan, 2000). On the other hand in falcons (Bailey, 2002), 5 µl semen has been mixed with 50 µl of a

TABLE 3 Pairwise comparison of the different manually evaluated live/dead stains via correlation analysis, matched *t* test for dependent samples and Bland–Altman analysis in semen samples of large falcons

		Correlation analysis		*t* test		Bland–Altman analysis	
	n	*r*-value	*p*-value	Mean ± *SD*	*p*-value	Regression curve	*p*-value
EB-EB2[1]	25	**.723**	**<.001**	**3.3 ± 5.1**	**.010**	y = 25.3 − 0.2 * x	.209
EB-EBN[1]	25	**.479**	**.033**	**−5.5 ± 5.2**	**<.001**	**y = −91.7 + 0.9 * x**	**.001**
EB-EYN5[1]	25	**.653**	**.015**	**−5.0 ± 5.2**	**.005**	y = −57.4 + 0.6 * x	.053
EB-EYN10[1]	25	.273	.244	**−4.9 ± 6.7**	**.004**	y = −27.7 + 0.2 * x	.503
EB-EBA[1]	25	.150	.529	**5.9 ± 15.5**	**<.001**	**y = 128.3 − 1.3 * x**	**<.001**
EB-EYA[1]	25	.071	.765	**7.2 ± 16.0**	**<.001**	**y = 142.0 − 1.5 * x**	**<.001**
EB-BBN[1]	25	.123	.606	**−4.9 ± 7.0**	**.005**	y = −51.7 + 0.5 * x	.235
EB-SYBR-PI[2]	33	**.346**	**.048**	**5.5 ± 15.5**	**.048**	y = 39.4 − 0.4 * x	.100

Abbreviations: BBN, Bromophenol blue–nigrosin; EB, eosin blue; EB2, eosin blue plus 3% sodium citrate; EBA, eosin blue–aniline; EBN5, eosin blue with 5% nigrosin; EYA, eosin yellow–aniline; EYN10, eosin yellow with 10% nigrosin; EYN5, eosin yellow with 5% nigrosin; mean, arithmetic mean; *n*, number of samples; *SD*, standard deviation; SYBR-PI, SYBR Green–propidium iodide.

Superscripts indicate whether the assessment was done in the first (1) or second (2) part of the study.

Bold values represent statistically significant observations.

nigrosin 5% –eosin blue 1%–Lake's diluent solution and incubated for two minutes on ice as done in mammals (Dott & Foster, 1972). Therefore, the results and statements in the present study are only valid for the stains and protocols used in the study.

Using eosin blue, the differentiation of dead from alive sperm was effectively possible, as reported from viability analysis in other avian species (Behncke, 2002; Fischer, Neumann, Purchase, et al., 2014; Lierz et al., 2013; Neumann et al., 2013; Schneider et al., 2018, 2019; Stelzer et al., 2005, 2009). Staining of defect cells was distinct and clearly visible in contrast to the bright background. In comparison with the other stains used in this study, EB offered a clear differentiation of alive and dead cells besides SYBR-PI. Similar results have been demonstrated in cockatiels recently, suggesting eosin blue and SYBR-PI as suitable live/dead stains (Schneider et al., 2017, 2018). The addition of sodium citrate (EB2), as suggested in some studies (Behncke, 2002), did not improve the staining properties for viability analysis. In contrast, some areas of the EB2 semen smears were stained too intense, altering the differentiation of dead cells from the background. Thus, the standard mixture EB (Fischer, Neumann, Wehrend, et al., 2014; Neumann et al., 2013; Stelzer et al., 2005) seemed to be better suited for sperm viability analysis compared with EB2.

Moreover, the identification of spermatids, pleomorphic sperm cells (Villaverde-Morcillo et al., 2017) and round bodies (Bailey et al., 2008; Hartley, 1998), a common finding in semen of raptors (Villaverde-Morcillo et al., 2017), was enabled in EB smears too. This offered, besides sperm viability analysis, the opportunity of subsequent sperm morphology analysis using EB stains. Identification of such pleomorphic cells was difficult, not possible or limited (cells were visible in reduced number only) using other live/dead stains in the semen samples of the present study (EBN5, EYN5, EYN10). Likewise, small and narrow sperm structures such as tail and acrosome were not identifiable using these stains. This demonstrated a clear disadvantage of other stains in the semen of large falcons regarding the usability for sperm viability and for sperm morphology analysis in accordance to descriptions in semen of non-raptorial bird species (Łukaszewicz, Jerysz, Partyka, & Siudzińska, 2008). Despite the pleomorphic cells with their specific staining properties, staining artefacts, overlapping of the background stain or inferior/weaker penetration of the staining combinations may explain the poor results in contrast to EB.

In summary, our results correspond to previous reports and underline the limited usability of eosin–nigrosin (Bailey, 2002; Blanco et al., 2008; Hartley, 1998; Madeddu et al., 2009) and of eosin aniline dye combinations in semen of raptors (Bailey, 2002; Bailey et al., 2008). Besides the limited usability for sperm viability analysis, EYA and EBA offered a good background contrast to spermatozoa and therefore potential benefits for sperm morphology analysis, which was not examined in the present study.

Comparison of viability results using the different live/dead staining protocols revealed that EB correlated significantly with EB2, EBN, EYN5 and SYBR-PI; even though total values of all stains, represented by arithmetic means, differed also significantly from EB. Bland–Altman analysis revealed that these differences increased in the lower viability region. In contrast, if TOV and OSV were high, none of the dyes should penetrate and colour sperm cells and thus difference should be less obvious.

To validate sperm viability, sperm motility values were correlated to sperm viability as previously described in roosters and pigeons (Chalah & Brillard, 1998; Klimowicz-Bodys et al., 2012). In the present study in falcons, EB was the only stain which correlated significantly and strongly to the amount of motile and progressively motile spermatozoa, with high viability values associated with high motility values. Statistically, SYBR-PI did also correlate significantly to MOT and PMOT in falcon semen, but this correlation using the Pearson coefficient was only very weak ($r < .5$). Therefore, our results in falcon semen differ from results in pigeons and chickens, as SYBR-PI and EYN correlated both significantly and strongly with sperm motility in semen of these

species (Chalah & Brillard, 1998; Klimowicz-Bodys et al., 2012). In particular, SYBR-PI has been shown to be more effective for viability count than EYN in fresh and frozen-thawed semen with a higher percentage of dead spermatozoa in fowl semen post-thawing (Chalah & Brillard, 1998) and in stored semen of pigeons (Klimowicz-Bodys et al., 2012). Some variation and falsification of correlation results in the falcon ejaculates may be caused by spermatids and round bodies, which have been demonstrated to be highly pleomorphic in falcons (Villaverde-Morcillo et al., 2017). However, identifiability of these cells differed between the various stains (e.g. different numbers of identified cells) which may cause additional differences in this regard.

In conclusion, EB seemed to be most suitable live/dead stain in falcons, showing significant and strong correlations to sperm motility.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Dominik Fischer performed the semen analysis, the laboratory work and the data analysis, wrote the manuscript and contributed to the study design. Helena Schneider performed the semen analysis and the laboratory work and revised the manuscript. Klaus Failing performed the statistical analysis and revised the manuscript. Axel Wehrend, Sabine Meinecke-Tillmann and Michael Lierz advised in the fieldwork, revised the manuscript and planned the study. All authors approved the final version of this article.

DATA AVAILABILITY

The authors confirm the absence of shared data and adherence to the policy.

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