Semen biotechnology optimization for successful fertilization in Japanese quail (*Coturnix japonica*).

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Short title: Japanese quail successful insemination conditions

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ABSTRACT

Among the reproductive biotechnologies needed to improve Japanese quail conservation and valorization, optimized conditions of semen methodologies including sampling, treatment, and artificial insemination are a prerequisite. However, they have been poorly developed due to specific physiological and behavioral features of the species. The aim of the present study was to optimize them, from semen collection/treatment up to artificial insemination procedures. We studied different parameters including semen preparation (individual/pooled, presence of foam, type and pH of extender) and zootechnical parameters (depth of insemination in the female tract, number of sperm inseminated, insemination frequency). We showed that the separation of semen from individual males was required to optimize fertility, as a prerequisite for future semen cryopreservation. The deleterious effect of mixed foam extract addition on the fertility level was demonstrated. These results highlight parameters involved in male copulatory competitions and in sperm post copulation selection. Furthermore, we took into account extender effects and standardized the zootechnical conditions of insemination. The depth of intravaginal insemination (1 cm) was a key factor, but not the number of sperm inseminated (15-60 million sperm/female). Finally, artificial inseminations with optimized conditions led to successful fertility rates (up to 80%) and a duration of the fertile period equivalent to that obtained by natural mating.

KEY WORDS: Japanese quail, artificial insemination, fertility, sperm

1. INTRODUCTION

Reproductive biotechnologies are important tools for genetic conservation, management of animal genetic diversity programs, development of farmed species, and new tools for research in model species. Quails are different bird species from the Phasianidae family in Asia and Europe and the Odontophoridae family in America, living in highly different places throughout the world. They have different statuses: wild/hunting species, farmed animals for egg and meat production, and model species for research. The Japanese quail (*Coturnix Japonica*) is the main domestic quail. Despite the high interest in developing reproductive biotechnologies in Japanese quails, very little progress has been made in this area due to specific physiological features of the species.

Quails are encountered as wild species in many Asian/European/American countries in different countryside biotopes with mild climates. Crossing between local wild quails and farmed quails may also occur, resulting in changes in wild genetic genomes. Japanese quails are also a farmed species bred for eggs and meat production. In comparison to other poultry productions, quails have many advantages: small animal size, low cost of breeding, early sexual maturity (6 weeks in natural spring conditions), short egg incubation time (17-18 days), and rapid generation turnover (4 generations per year). Their lay rate may be extremely high, more than 300 eggs/female [1, 2].

The Japanese quail is also an avian model species for research. It is used as a laboratory research animal for avian genetics, nutrition, behavior, neuroendocrinology, and physiology studies [3-8]. Both embryos and adult Japanese quails are models in the study of human diseases [9] or ageing[10]. The Japanese quail genome was sequenced in 2013 [11]. This opens up the opportunity to use this species for the production of transgenic birds [12].

Among the reproductive biotechnologies needed to improve quail conservation, management, and dissemination, successful semen sampling, treatment, and artificial insemination are a prerequisite. The development of these methodologies must take into account the specific reproductive physiology and behaviors of this species. Briefly, at sexual maturity, Japanese quail males produce daily a small amount of sperm (10-15µl on average and 600 x 10^6 to 5 x 10^9 sperm per ml; [1, 13]) compared to other domesticated avian species such as chickens and turkeys [13, 14]. Sperm morphology and metabolism also seem to be quite specific since sperm length is much longer (250 µm) than in chickens and turkeys (90-100 µm), and contains a much higher number of mitochondria (more than 1,400 compared to 30 for the chicken) [15, 16]. Another specificity of the Japanese quail male is the presence of foam, a non-semen copulatory fluid, produced by the proctodeal glands [17]. During natural copulation, the foam was reported to increase fertility and to extend the fertility duration [17-19]. After artificial insemination, the foam was characterized as a mobility enhancer and sperm agglutination reducer, but fertility remained low (< 40%) [20, 21]. *In vitro* studies of foam addition into semen extender showed a positive impact of 5 to 10% foam extract on the motility of sperm, while higher levels (≥ 15%) suppressed sperm motility [22, 23]. Foam might play a role in the post-copulatory sperm competition between different males and to secure the fertilization obtained with the sperm of a given male [24]. The storage of sperm in the female sperm storage tubules (SST) has been reported to be much shorter (mean 10 days) than in chickens (18-21 days) and turkeys (2-3 months) [13, 25]. Male sexual behavior is very marked with high copulation frequencies in the presence of females or even males [5, 26]. In addition, in breeding conditions, quails often remain highly stressed and need to be very carefully managed. Considering these features, and despite their importance for further semen conservation and other biotechnology developments, artificial insemination methodologies have been poorly developed in this species and still result in highly variable success [27-29].

The present study was thus conducted in order to optimize the semen collection/treatment and artificial insemination procedures, a prerequisite for further biotechnical developments in Japanese quails. We studied different parameters including the use of pooled semen (from different males) or individual semen, dilution, extender pH, presence or absence of homologous or heterologous foam, depth of insemination in the female tract, number of sperm inseminated and frequency of insemination. We highlighted that the separation of semen from individual males was required to obtain good fertility, which may be due to sperm competition between males. Furthermore, we standardized the zootechnical conditions of insemination: extender, best insemination dose, depth and frequency in order to obtain the best fertility rate.

2. MATERIALS AND METHODS

2.1. Housing and management

Animals were housed at the INRA experimental unit UE-PEAT in Nouzilly (France) following the European welfare and the French Direction of Veterinary Services regulations (agreement number C37-175-1). Hatching eggs were obtained from commercial stock (Robin, Maché, France). After hatching, male and female quails were housed together up to the age of 8 weeks under 24H light the first days and then low photoperiod (8D/16L, a photoperiod that does not stimulate gonad growth). They were then housed in individual battery cages (for males) or by groups of two (for females), according to European Welfare regulations regarding animal experimentation, under a 14L/10D photoperiod (gonad and sexual stimulating photoperiod) and fed *ad libitum* with a standard diet. In order to reduce the birds' stress to human voices and all extra noises, radio programs (music, human voices) were

diffused in the rooms from birth. Experiments were always performed with the same technical support and people to limit technical and human effects.

2.2. Semen collection

Semen was routinely collected twice a week using a teaser female as previously described [1] with some adaptations. The female was placed in the male's cage and at the time of intense excitation, the male was taken out and placed on the back in a restraint system. Cloacal glands were voided of foam and, after a gentle squeezing of the spermatic glands, semen was collected from the everted copulatory organ using a micropipette and a cut cone. Sperm concentration was immediately determined by light absorption of the semen with a photometer (Accucell photometer, IMV Technologies, L'Aigle, France) at a wavelength of 530 nm [30]. The semen volume varied from 5 to 80 µl with a mean of 40 µl, and the mean sperm concentration was 4 x 10^9 sperm/ml. Ejaculates (pooled semen or individual ejaculate) were then used directly for analysis or female insemination.

2.3. Semen evaluation

Semen from individual or pooled ejaculates was analysed for motility and viability criteria. Objective measurements of motility were evaluated using the computer-assisted sperm analysis (CASA) system with an HTM-IVOS (Hamilton-Thorn Motility Analyzer, IVOS). In this experiment, the parameter measured was the percentage of motile sperm (%). Quail semen was diluted in MB at 39°C (dilution 1:1). Sperm concentration was adjusted to 20 x 10^6 sperm/ml. The motility was evaluated by counting a minimum of 200 sperm per ejaculate in a Makler cell chamber prewarmed at 39°C.

Sperm viability was assessed with propidium iodide (PI) fluorescent dye. Aliquots from each sample were adjusted to a final concentration of 2 x 10^9 cells / ml with MB. Then, sperm were

diluted in Lake 7.1 buffer down to 2 x 10^7 cells / ml and 2 µL of PI was added. Resulting samples were incubated for 15 min in the dark at room temperature. Sperm viability was then assessed with a Guava easyCyte Flow Cytometer (IMV Technologies, L'Aigle, France). A total of 5,000 sperm per sample were analyzed.

2.4. Foam collection and preparation of foam extract

After excitation of a male with the teaser female, proctodeal gland foam was collected by gently squeezing either side of the proctodeal gland with fingers and foam extract was prepared as previously described [23]. Briefly, foam collected from individual or pooled males (n = 10) was mixed with saline solution (0.89% wt/vol NaCl) in a 1:2 ratio followed by vortexing for 30 min. The mixture was centrifuged at 10 000 g for 30 min and the supernatant was considered to be a foam extract containing 33.3% foam. The 10% foam extract was prepared by diluting the foam extract (33.3%) with the motility buffer (MB), which is a NaCl-TES buffer supplemented with 35 mM of glucose and 0.1% of BSA, pH 8.0 and 330 mOsm [23].

2.5. Female insemination and fertility measurements

The time between the start of semen collection and the end of the insemination process was 15 min maximum. Two females/male were inseminated with sperm from the different conditions (sperm number specified in the experimental design section, Table 1) in the vagina with a pipette provided with a cut cone to avoid injury. Inseminations were performed 3 hours after the daily lay in order to avoid contact with the uterine egg and to optimize AI conditions. Inseminations were repeated four times at several days of interval for experiments 1 to 6. Eggs (150 to 300/treatment) were collected daily, starting on the second day after the first insemination, fumigated and stored at low temperature in the experimental hatchery. Every week, all eggs were set in the incubator and fertility rate (% fertile/incubated eggs) was

measured by candling after 7 days of incubation. For each condition (experiments 1 to 6), the mean fertility rate was calculated by averaging the fertility rates of days two to four after insemination.

2.6. Experimental design

Experimental conditions of the experiments 1 to 6 are summarized in Table 1. Briefly, in experiment 1, semen was pooled (n = 7 males) or used individually and diluted 1:1 or not in the motility buffer (MB) at pH 8.0. In experiment 2, individual ejaculates were diluted 1:1 in MB buffer containing or not 10% of foam extract from pooled foam (n=10 males) or individual foam corresponding to the ejaculate. In experiment 3, individual semen was diluted 1:1 in MB buffer at pH 7.4 or 8.0. In experiment 4, the intravaginal depth of insemination was tested (1 cm or 2 cm). In experiment 5, the sperm number inseminated (individual ejaculate diluted 1:1 in MB buffer pH 7.4) was tested (15, 30 or 60 x 10^6 sperm/female/AI). In experiment 6, frequency of insemination was evaluated (high frequency, every 3 days or low frequency, 1 time per week).

2.7. Duration of the fertile period (DFP) and useful duration

Duration of the fertile period was determined by natural mating or after artificial insemination. For natural mating, one male and two females were put together for two days. Eggs were collected daily from day 2 to 14 after male removal. The fertility rate was estimated as described above. Duration of the fertile period was also determined after a single artificial insemination (individual male semen, diluted 1:1 in MB at pH 7.4, 1 cm depth insemination, 60 x 10^6 sperm/female). Eggs were collected daily from day 2 to 14 after insemination. The fertility rate was estimated as described above by egg candling after 7 days of incubation.

2.8. Statistical analysis

Statistical analysis for multiple comparisons was performed using the nonparametric Kruskal-Wallis test follow by Tukey's HSD. Percentages were transformed into arcsine square-roots before analysis. The data were plotted with the boxplot module of R graphics library (http://cran.r-project.org; R version 3.5.1). The module displays the median, a box formed by the quartiles, the range and outliers of the data. The *P* values were calculated on the null hypothesis (no shift in the data distributions of the compared groups). The level of significance of the differences between experimental conditions (fertility rate, motility rate, or viability rate) was set at a *P* value of 0.05.

3. RESULTS

3.1. Individual semen is required for fertility improvements (experiment 1)

We tested the impact of individual ejaculates or pools from 7-8 males (individual vs pooled semen, Table 1) on sperm viability, motility, and fertility rates obtained after artificial insemination. Sperm motility (mean percentage of motile sperm 42.3% and 37.1%, respectively, p = 0.143) and viability (96.8% and 94.6%, p = 0.117) were not significantly different between the treatments (Fig 1 A, 1B). The results reported in Fig. 1C and Table 2 show that individual semen (gray boxes) provided significantly higher fertility results than pooled semen (white boxes) (30.6% and 19%, respectively, p = 0.048). The dilution in MB did not significantly affect the fertility obtained with individual ejaculates (30.6% and 24.5%, respectively, p = 0.51), but further decreased, although not significantly, the results (already lower) obtained with pooled semen (19.0% and 10.1%, respectively, p = 0.18).

3.2. Foam extract supplementation has a negative effect on fertility rate (experiment 2)

We inseminated females with individual ejaculates supplemented with foam extract or not (Table 1 experimental design; Fig.2 and Table 2 fertility results). The foam was collected and prepared from individual males (corresponding to males used for AI) or pooled semen. The individual semen collected without foam showed the highest fertility results (52.7%) without a significant difference with the individual semen supplemented with 10% of their homologous foam extracts ($p = 0.304$). Moreover, the addition of pooled foam decreased the fertility rate ($p = 0.087$). Our results show that foam was not essential for insemination success. For following experiments, foam was not added in the extender.

3.3. Extender pH effect (experiment 3)

We compared the influence of the MB extender at pH 7.4 or 8.0 on fertility rate after individual insemination. An aliquot of undiluted semen was the control. Results are presented in Fig. 3 and Table 2. Fertility was not significantly affected ($p = 0.069$) by the extender and its pH despite a tendency of negative effect of pH 8.0 (39.9% and 19.3% for pH 7.4 and 8.0, respectively; high individual variability between males). We decided to keep the extender at pH 7.4 for diluting sperm samples.

3.4. The depth of intravaginal insemination is crucial for fertility (experiment 4)

We then tested the impact of the intravaginal insemination depth on fertility (Table 1). Results obtained after an artificial insemination depth at 1 or 2 cm are shown in Fig. 4 and Table 2. The fertility rate was twice as high when the insemination was made not deeply (57.5% vs. 23.3%, 1 cm and 2 cm, respectively; $p \leq 0.0007$). We thus suggest inseminating at a maximum intravaginal depth of 1 cm.

3.5. The number of sperm is not crucial for the success of AI (experiment 5)

We tested three insemination doses: 15, 30 or 60 x 10^6 sperm/female (Table 1). The results are reported in Fig. 5 and Table 2. We showed no significant effect of the insemination dose in the range 15 to 60 x10^6 sperm/IA/female ($p = 0.094$). Since there was a tendency in favor of the dose 60 x 10^6 sperm when compared to 30 ($p = 0.091$), we suggested keeping the dose 60 x10^6 sperm as the standard AI dose.

3.6. Insemination frequency (Experiment 6)

The effects of the insemination frequency (1 or 3 times a week, Table 1) on the fertility rate are shown in Fig. 6 and Table 2. Repeated inseminations (every 3 days) led to higher fertility results than a weekly insemination (81.4% and 66.1%, respectively) ($p \leq 0.007$).

3.7. Duration of Fertile Period: natural mating versus artificial insemination

Duration of the fertile period was determined by natural mating (Fig. 7, black line) or after a single insemination of diluted fresh semen (Fig. 7, gray line). Japanese quails were able to store sperm for 10 days after natural mating. After artificial insemination with our optimized conditions, the duration of the fertile period is similar to natural mating, proving that our conditions (sperm preparation and zootechnical skills) are suitable for Japanese quail artificial insemination success.

4. DISCUSSION

In the present study, we showed how specific features of quail reproductive physiology might be efficiently taken into account in order to optimize semen management for high fertilization success after artificial insemination. We showed that the separation of semen from individual males was required to optimize fertility, and that mixed foam extracts

were deleterious. These results highlight features involved in male copulatory competitions and in sperm post copulation selection. Furthermore, we took into account the extender effects and standardized the zootechnical conditions of insemination: best insemination dose, depth ,and frequency to obtain a successful fertility rate (up to 80%).

Quails are birds of small size compared to other domestic birds. Their amount of semen delivered at ejaculation remains low even if we could increase semen collection in the present study (up to 80 µl/ejaculate, with a mean at 40µl, and mean sperm concentration of 4 x 10^9 sperm/ml in our present work). A foam of much higher volume (more than 10^3 higher volume than semen, mean weight 20 mg [22]) is produced by the proctodeal gland of the cloaca at the time of ejaculation. This foam is suspected to play a key role in reproduction [17-19, 23]. Quails are also highly sensitive animals and ejaculation is more efficiently obtained in the proximity of females. Since the 1960s, different studies have been carried out to develop Japanese quail artificial insemination. While fertility was often very high by natural mating (frequently more than 90%) [17], the fertility rate obtained after artificial insemination did not exceed 70% [27, 28]. In the last decade, new experiments were done to understand the fertilization process and increase the fertility rate. The results obtained were very variable (9% to 66%) depending on the protocol used (dilution of sperm or not, addition of foam or not, etc.) [19, 29, 31, 32]. Quails are very sensitive and easily stressed animals. Fluctuations of AI success due to punctual events (i.e. an unexpected noise) may never be totally removed. However, we show in the present study that different factors of variations may be efficiently controlled.

For artificial insemination practices, the question of the success of potential fertilizing ability of individual semen or of semen pooled from different males (which could be easier to manage) is still a key point for the success of semen biotechnologies. Since the foam is impossible to remove completely from semen at ejaculation even when foam is manually

279 discarded, the question of the presence of homologous foam (from an individual male if
280 individual semen is used for AI) or of heterologous foams (if semen pooled from different
281 males is used) is open to exploration. Interestingly, to our knowledge, no study had been
282 performed using semen from individual males, perhaps due to the small volume (20-30µl per
283 ejaculate) of collected semen [1].

284 We clearly showed in the present study that the use of semen from individual males
285 gave higher fertility results than the use of semen pooled from different males. We also
286 showed that the addition of 10 % mixed foam is harmful. The exploration of the hypothesis
287 able to explain these observations lead to the suggestion that the foam shares specific features
288 that make it unfavorable to artificial insemination practice if mixed, thus explaining why
289 mixed semen (with residual amounts of mixed foams) is less efficient than individual
290 ejaculates. For that reason, individual semen, (with traces or with 10% of its own foam)
291 provides a better guarantee of success of Artificial Insemination. The foam may be a specific
292 sexual attribute whose role is not clearly elucidated [19, 24, 33]. During natural mating, the
293 foam seems to help maintain sperm inside the female genital tract and to stimulate egg
294 penetration [17, 18]. It stimulates *in vitro* sperm motility [19, 22, 23], and sperm transport in
295 the female tract [19], but may decrease sperm viability [29]. Positive effects on fertility were
296 seen only in cases of use of mixed semen and comparison with dilution in simple saline
297 solutions [19]. Few data are available about the biochemical composition of foam [34, 35]. A
298 role in energy metabolism of quail sperm was suggested, due to the presence of proteins,
299 lactate, and metabolism enzymes commonly found in the blood [35]. Lactate was considered
300 to be the main foam metabolite that leads to *in vivo* and *in vitro* stimulation of sperm motility
301 [19, 21, 35].

302 The presence of the foam of one male had also been suggested to decrease the
303 fertilization success of a rival by decreasing the efficiency of additional copulations of a

304 female with other males [24]. Thus, foam could function as a biochemical barrier for post-
305 copulatory selection in the female genital tract. This could explain why, in our present study,
306 the use of foam from individual males still give better fertility results than the use of foam
307 from different males

308 The effect of the diluent may also be an important factor of success. Previous studies
309 with pooled semen showed that semen dilution at 1:1 showed contrasted effects on the
310 fertility rate [29, 31, 36]. We observed in the present study, with the use of individual semen,
311 that semen dilution at 1:1 ratio (semen:extender) in NaCl-TES extender supplemented with 35
312 mM glucose and 0.1% BSA (MB buffer) did not significantly affect the fertility rate, despite a
313 slight decline (30.6% and 24.5%, respectively, $p = 0.51$).

314 The pH of the diluent seems to play a role in the motility of the quail sperm. It was
315 demonstrated that motility is inhibited when the pH is below to 7.2 and then increases
316 progressively when the pH is increased until pH 8.0 [37], The pH 8.0 was considered to be
317 optimal for sperm motility [23]. We chose to work with a collection buffer at pH 7.4 because
318 the pH of the quail semen was 7.32 (data not shown). In our study, the effect on fertility of the
319 pH extender was not significant in the range 7.4-8.0 but the higher pH showed a tendency to
320 be less efficient than 7.4 ($p = 0.18$). These results show once again that fertility results may
321 significantly differ from sperm motility results.

322 Optimal semen collection and preparation seem to be essential for the success of
323 fertilization after artificial insemination. Zootechnical skills are very important, as well as
324 experience, as demonstrated by our fertility results that increased with time and expertise,
325 from 30% (figure 1) to more than 80% (figures 6, 7). The place of the insemination in the
326 female tract is another factor to take into consideration. Previous results had shown that intra-
327 peritoneal or intra-uterine inseminations were less successful than intravaginal inseminations

328 [27, 29]. Before our results, the effect of the intravaginal insemination depth was unknown
329 [28, 29]. We showed, surprisingly, that the depth of intravaginal insemination is crucial since
330 the fertility rate decreased 2.5-fold when the depth was changed from 1 to 2 cm. We
331 hypothesize that this optimal depth of 1 cm must take into account the vagina length in order
332 to avoid proximal-uterine inseminations which could hypothetically be more stressful or could
333 lead to a less efficient sperm flow into the Sperm Storage Tubules (SSTs). In contrast, a too
334 short introduction of sperm into the vagina might lead to further rejection of semen by the
335 female. The vaginal length that we measured in "Robin" Japanese quail females was a mean
336 of 3 cm (unpublished observation). This is substantially shorter than in bigger birds such as
337 hens or turkeys (a mean of 5-8 cm in many hens). We may consider that an intravaginal
338 insemination in the first half of the vagina (1 cm in the "Robin" Japanese quails of the present
339 study, a mean of 3 cm in domestic hens) generally seems to be a good compromise to
340 optimize AI. Of course, the vaginal length may vary among the breeds within a particular
341 species and future experimental adaptations would also take into account this factor.

342 Another factor that we thought would be important and that had not been investigated
343 before was the number of inseminated sperm. Surprisingly, we showed that the number of
344 sperm inseminated was not so critical in the range 15 to 60 million
345 sperm/insemination/female. This could mean that the capacity of the SSTs of the female quail
346 to store sperm could be more limited than expected. In our study, a quite high fertility rate
347 (~80%) could be maintained after twice a week intravaginal inseminations at the depth of 1
348 cm (figure 6). We noticed that, with the best conditions, the duration of the fertile period after
349 a single AI is similar to after natural mating (10 days). The duration of the fertile period was
350 previously estimated to be 8-10 days after natural mating of a quail pair [26]. The decrease in
351 fertility with increasing days after mating was correlated with a decrease in the number of
352 sperm stored in the SSTs [38]. According to our results on the poor effect of different semen

353 doses on fertility, our results of fertility duration suggest that the SST of the Japanese quail
354 are not filled by the artificial insemination in our conditions. One hypothesis is that, during
355 natural mating, there are many copulations in a short time, thus the SSTs are expected to store
356 a higher number of sperm than after a single AI. It has been previously described that the
357 male produced prostaglandins F2α (PGF2α) in the cloacal foam that is suspected to be
358 transported into the vagina at the time of mating, and to support sperm uptake into the female
359 quail SST [39].

360 Altogether, in the present study, improvements in semen collection (individual
361 ejaculate), treatment and artificial insemination procedures (depth of insemination of 1 cm,
362 frequency every 3 days) lead to a standardized protocol with which we obtained a fertility rate
363 of more than 80%. This standardization is the first prerequisite for the development of
364 reproductive biotechnologies needed to improve quail conservation through semen
365 cryopreservation, management, and dissemination.

366

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373

374 **CONFLICT OF INTEREST**

375 The authors declare that they have no conflicts of interest.

376

377 **FIGURE LEGENDS**

378 **Figure 1A.** Evaluation of motility for semen collected from individual males (in gray) or
379 pooled (in white) with MB buffer dilution. Different superscripts indicate significant
380 differences between treatments ($P < 0.05$).

381 **Figure 1B.** Evaluation of viability for semen collected from individual males (in gray) or
382 pooled (in white) with MB buffer dilution. Different superscripts indicate significant
383 differences between treatments ($P < 0.05$).

384 **Figure 1C.** Initial evaluation of conditions for semen preparation for artificial insemination:
385 semen collected from individual males (in gray) or pooled (in white) with or without MB
386 buffer dilution. Different superscripts indicate significant differences between conditions ($P <$
387 0.05).

388 **Figure 2.** Effect of addition of foam from one male (indiv) or pooled males (pooled) on
389 fertility rate after artificial insemination. Different superscripts indicate significant differences
390 between conditions ($P < 0.05$).

391 **Figure 3.** Optimization of the pH of the MB buffer used for semen dilution. Different
392 superscripts indicate significant differences between conditions ($P < 0.05$).

393 **Figure 4.** Fertility levels after artificial insemination at 1 cm or 2 cm depth. Different
394 superscripts indicate significant differences between conditions ($P < 0.05$).

395 **Figure 5.** Effect of the dose of semen inseminated (15, 30 or 60 x 10^6 sperm per female) on
396 fertility rate. Different superscripts indicate significant differences between conditions ($P <$
397 0.05).

Figure 6. Influence of frequency of insemination on fertility levels. Different superscripts indicate significant differences between conditions ($P < 0.05$).

Figure 7. Comparison of duration of fertile period after natural mating (black line) or a single artificial insemination in our standardized conditions (gray line).

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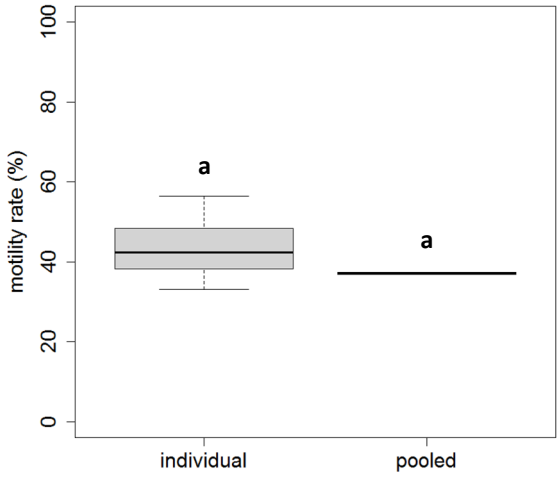
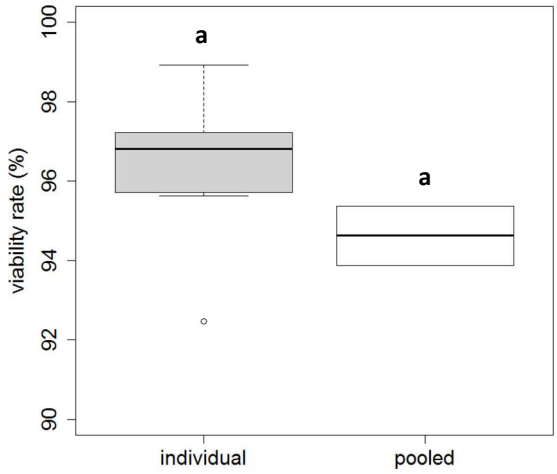
498

Figure 1A.



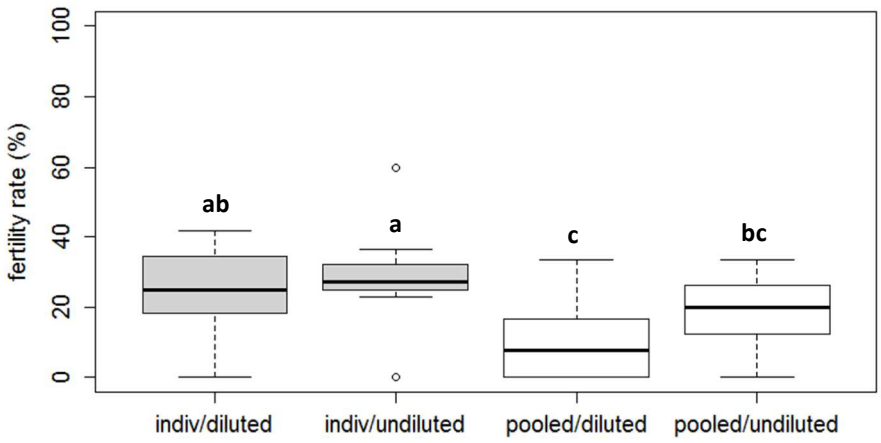
Evaluation of motility for semen collected from individual males (in gray) or pooled (in white) with MB buffer dilution. Different superscripts indicate significant differences between treatments ($P < 0.05$).

Figure 1B.



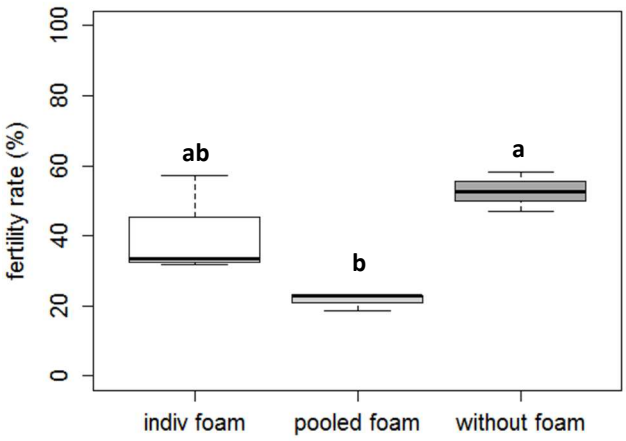
Evaluation of viability for semen collected from individual males (in gray) or pooled (in white) with MB buffer dilution. Different superscripts indicate significant differences between treatments ($P < 0.05$).

Figure 1C.



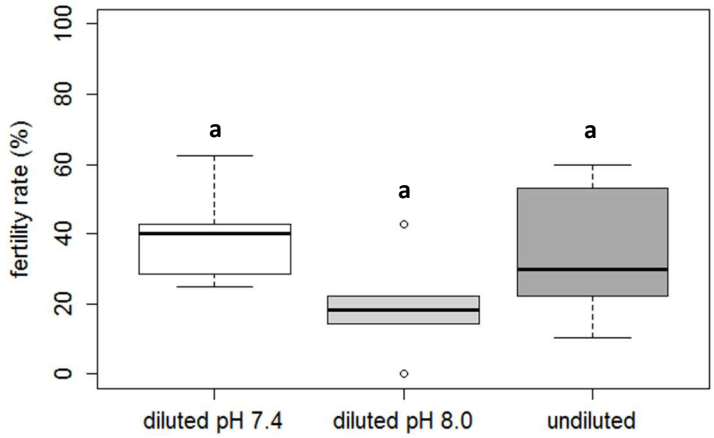
Initial evaluation of conditions for semen preparation for artificial insemination: semen collected from individual males (in gray) or pooled (in white) with or without MB buffer dilution. Different superscripts indicate significant differences between conditions ($P < 0.05$).

Figure 2.



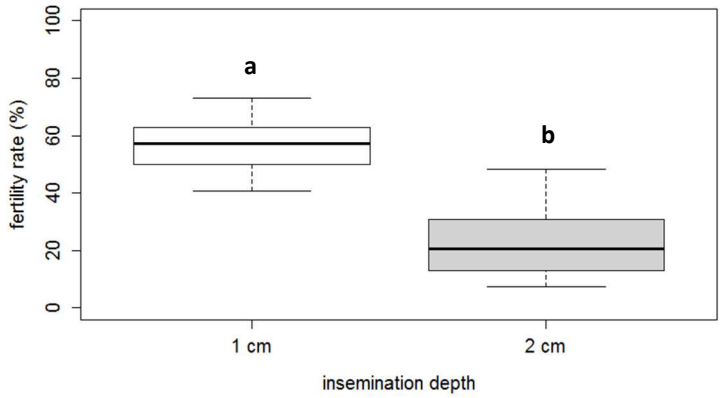
Effect of addition of foam from one male (indiv) or pooled males (pooled) on fertility rate after artificial insemination. Different superscripts indicate significant differences between treatments ($P < 0.05$).

Figure 3.



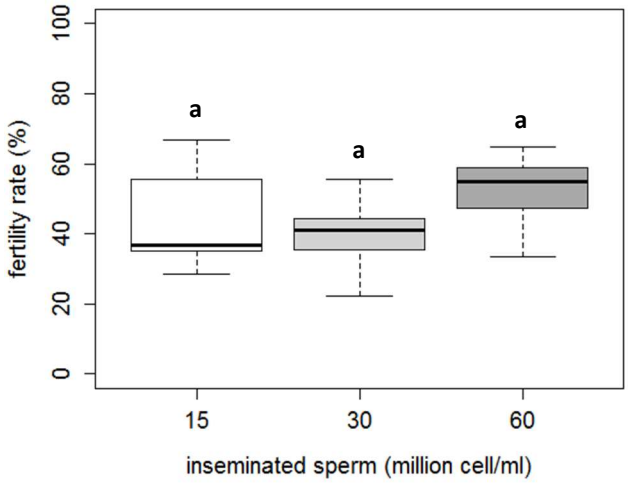
Optimization of the pH of the MB buffer used for semen dilution. Different superscripts indicate significant differences between treatments ($P < 0.05$).

Figure 4.



Fertility levels after artificial insemination at 1 cm or 2 cm depth. Different superscripts indicate significant differences between treatments ($P < 0.05$).

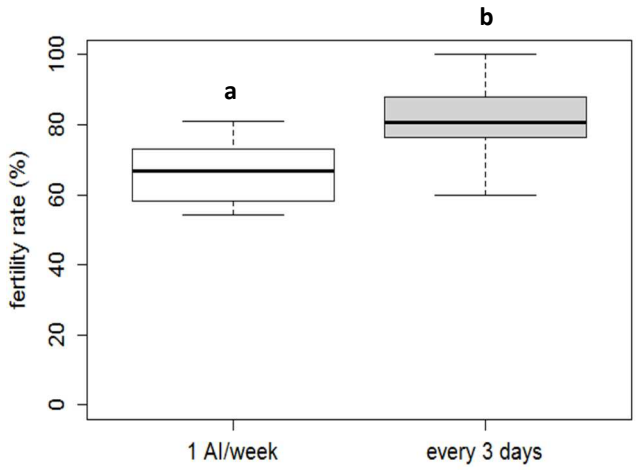
Figure 5.



Effect of the dose of semen inseminated (15, 30 or 60 x 10^6 sperm per female) on fertility rate.

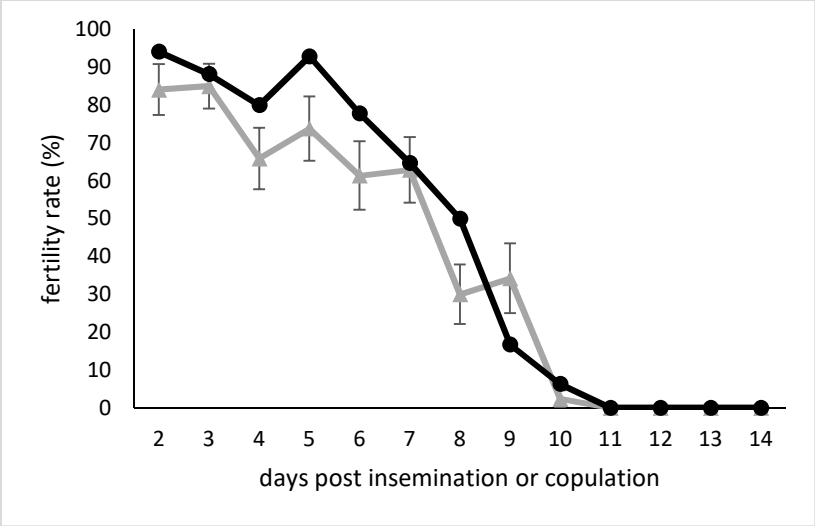
Different superscripts indicate significant differences between treatments ($P < 0.05$).

Figure 6.



Influence of frequency of insemination on fertility levels. Different superscripts indicate significant differences between treatments ($P < 0.05$).

Figure 7.



Comparison of duration of fertile period after natural mating (black line) or a single artificial insemination in our standardized conditions (gray line).

Table 1. Experimental design of the fertility measurements (experiments 1 to 6).

	age (weeks)	sperm (x 10^6 / female)	insemination frequency	insemination depth	number of males	number of females	number of incubated eggs
Experiment 1 : sperm pool and dilution							
individual / undiluted	20-22	15	every 3 days	2 cm	8	16	179
individual / diluted 1:1					8	16	188
pooled / undiluted					7	14	169
pooled / diluted 1:1					7	14	130
Experiment 2 : addition of foam							
individual with 10% individual foam	27-28	15	every 3 days	2 cm	15	30	216
individual without foam					13	30	192
individual with 10% pooled foam					14	28	168
Experiment 3 : extender pH							
pH 7.4	12-13	15	every 3 days	2 cm	4	8	
pH 8.0					5	10	
undiluted					11	22	186
Experiment 4 : insemination depth							
1 cm	35	30	every 3 days	-	25	50	245
2 cm					22	44	252
Experiment 5 : sperm per AI							
15 x 10^6	38	-	every 3 days	1 cm	16	32	160
30 x 10^6					16	32	156
60 x 10^6					15	30	160
Experiment 6 : insemination frequency							
every 3 days	12	60	-	1 cm	12	24	249
1 time / week					18	36	322

Age: age of the males and the females. Sperm: number of sperm inseminated/female. Number of males, number of females: respective number of males and females used for artificial insemination. Insemination depth: depth of the intravaginal insemination of sperm. Individual: ejaculate of individual male. Pooled: semen pooled from 7-8 males. Diluted: semen diluted in MB buffer.

Table 2. Summary of fertility rates (%) obtained for each experimental condition.

	fertility rate (%)
Experiment 1 : sperm pool and dilution	
individual / undiluted	30.6 ± 3.7[a]
individual / diluted 1:1	24.5 ± 2.9[ab]
pooled / undiluted	19.0 ± 2.4[bc]
pooled / diluted 1:1	10.1 ± 3.1[c]
Experiment 2 : addition of foam	
individual with 10% individual foam	40.8 ± 8.2[ab]
individual without foam	52.7 ± 3.3[a]
individual with 10% pooled foam	21.6 ± 1.4[b]
Experiment 3 : extender pH	
pH 7.4	39.9 ± 5.4[a]
pH 8.0	19.3 ± 5.8[a]
undiluted	34.3 ± 7.7[a]
Experiment 4 : insemination depth	
1cm	57.5 ± 3.5[a]
2cm	23.3 ± 4.4[b]
Experiment 5 : sperm per AI	
15 x 10^6	44.0 ± 4.4[a]
30 x 106	40.4 ± 3.3[a]
60 x 106	52.9 ± 3.3[a]
Experiment 6 : insemination frequency	
1 time / week	66.1 ± 2.3[a]
every 3 days	81.4 ± 2.9[b]